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Drug targeting to the kidney and to the urinary bladder

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Drug targeting to the kidney and to the urinary bladder

RIJKSUNIVERSITEIT GRONINGEN

Drug targeting to the kidney and to the urinary bladder

PROEFSCHRIFT

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Marijke Haas

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te Geldrop

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"humanized rats"

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Introduction

Strategy of drug targeting to the kidney and to the urinary bladder

Scope of the present study

This thesis covers the research field of "drug targeting to the kidney and to the urinary bladder". Drugs are designed to interfere with pathological conditions either to cure diseases or to alleviate symptoms of diseases. For instance, drugs can mimic the activity of endogenous substrates under conditions of substrate depletion or can antagonize endogenous compounds if overproduction occurs. However, drugs should not only be designed on the basis of their activity properties but also from the point of view of their disposition in the body. Directing a drug specifically to the desired site of action can not only increase the therapeutic effect but may at the same time reduce unwanted actions in non-target tissue. This strategy of so called "drug targeting" may be of interest to improve the therapy of certain diseases residing in the kidney and in the urinary bladder as reviewed in chapter I. For example, glomerular leakage of proteins results in kidney damage [1] and reduction of such proteinuria protects the kidney [2]. Drugs that are commonly used to reduce proteinuria are non-steroidal anti-inflammatory drugs (NSAIDs) and angiotensin-converting enzyme inhibitors (ACE-inhibitors). However, these drugs have certain non-renal (systemic) actions that, in most cases are unwanted. A specific delivery of such a drug to the kidney may prevent the drug to affect non-renal targets and offers the possibility to lower proteinuria more aggressively.
Because of the high systemic toxicity and low urinary excretion of drugs used for treatment of bladder disorders like cancer, local drug application has obvious advantages. A local administration procedure is currently used in practice: direct irrigation of the bladder with cytostatic agents prevents the rest of the body to be exposed. This mechanical method of drug delivery is however far from ideal, since it is inconvenient for the particular patients and often leads to cystitis. Besides, the procedure is time-consuming and laborious compared to other methods of administration [3]. Therefore, a systemic method of drug targeting to the bladder in which the drugs are given parenterally in a renal-selective formulation might be preferable.

Strategy of drug targeting to the kidney and to the urinary bladder

Figure I summarizes the different aspects that are involved in research on drug targeting to the kidney and to the urinary bladder. On the one hand, one has to consider the treatment options in relation to the (patho)physiology of the disease and on the other hand, the feasibility of drug targeting has to be taken into account.

Figure 1

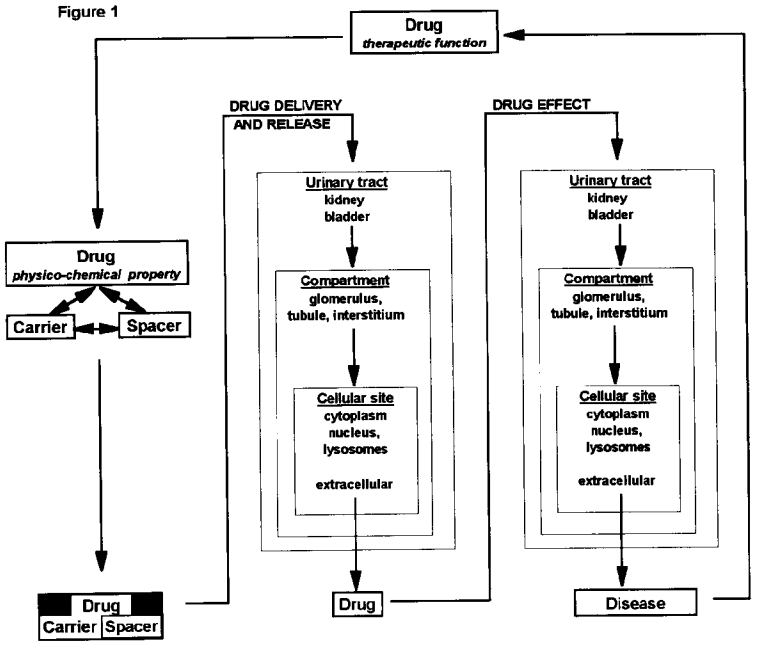

Table 1

Drug	**Diseases/ abnormalities**
Physico-chemical properties:	renal function loss
Functional group (amino-, thiol-, carboxyl group), lipophilicity, charge.	(glomerulo-, interstitial nephritis, recurrent infection etc.)
Carriers	proteinuria,
To the glomerulus:	transplant rejection,
liposomes (charge/antibody fragment modified), antibody/albumin (charge/size modified), cells	bladder/kidney tumor
To the proximal tubule or bladder:	**Drugs**
low-molecular weight proteins, polymers	***Therapeutic function***
Spacer	ACE-inhibitors, NSAIDs, anti-fibrotic agents, immune suppressants, anti-tumor drugs, genes,
Functional group (peptide, ester, thiol, acid-labile)	antisense oligo-nucleotides

Figure 1. *Design of a construct for drug targeting to the kidney or to the urinary tract.* Left panel: The drug must be coupled to (or incorporated in) a carrier. To allow drug-attachment and site-specific drug release, a spacer may be required between the drug and carrier.
Middle panel summarizes the different compartments in the kidney and urinary bladder that may be reached using a specific carrier. Depending on the compartment of drug delivery, a specific spacer may be required for drug activation.
Right panel: After activation of the drug at the site of delivery, the drug must reach its site of action in the kidney or urinary bladder.

Table 1. *Examples of drug carriers, physico-chemical properties and spacers that should be combined in the design of a drug-targeting preparation.*

With respect to the drug targeting strategy (left and central panel), a drug carrier should be chosen that delivers the drug to (or close to) the compartment where drug action should take place. Furthermore, the drug should be attached to (or incorporated in) the carrier in such a way that it can be released in its active form after reaching the target tissue. From this targeting point of view, the drug is not chosen for its dynamic properties but for physico-chemical reasons. A spacer moiety between the drug and the carrier may be required to enable proper drug release. Table I summarizes some examples of drug carriers, physico-chemical properties of drugs and spacers that could be combined in the design of a drug-targeting preparation.
With respect to the (patho)physiology (figure 1, right panel), one has to identify urinary tract diseases as well as the kind of drugs that are effective in treatment (Table I: Drug *therapeutic function*). Furthermore, it is helpful to have information on the compartment of the urinary tract in which the drug should exhibit its therapeutic activity.

Scope of the present thesis

Drug carrier: the low-molecular-weight protein
Low-molecular-weight proteins (LMWPs) are proteins smaller than about 20 kDa. They are predominantly catabolized in the kidney [4,5]. Because of their small size, they are freely filtered in the glomerulus. Subsequently, they are reabsorbed by the proximal tubular cell and are catabolized intracellularly in the lysosomes. By coupling a drug to such a protein, renal specific drug targeting may be obtained. The drug will thus be transported specifically to the kidney and will be released during catabolism in the lysosomes of the proximal tubular cell. Alternatively, studies have shown that tubular uptake of a LMWP after glomerular filtration can be prevented by shielding the positively charged groups on the protein leading to more complete excretion in the urine [6-8]. Drug targeting to the urinary bladder may thus be possible using such a modified LMWP as carrier. These concepts of drug targeting using LMWPs are visualized in figure 2.

The chapters 2, 3 and 4 describe the studies which were performed to extend the knowledge of the renal handling of LMWPs.

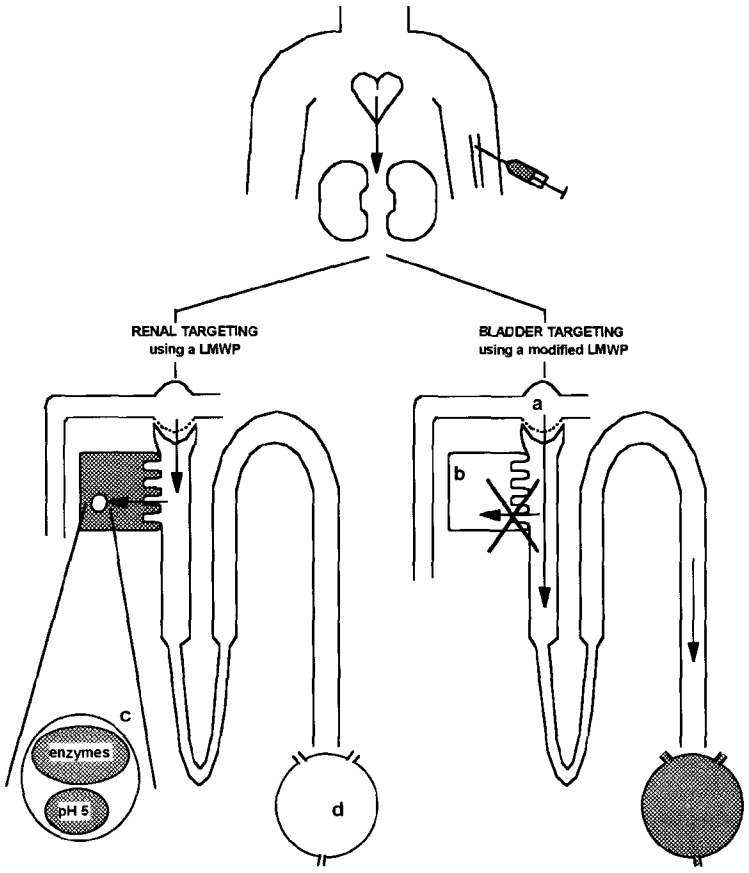

Figure 2
a: glomerulus, b: proximal tubular cell, c: lysosomes, d: urinary bladder

Chapter 2:
Research question: are LMWPs specifically taken up by the kidney?
- how much of an administered dose is taken up by the kidney?
- is there a difference in renal specificity between various LMWPs?

Application for drug targeting: the renal specificity of a LMWP will determine how much of a drug can be delivered to the kidney using that LMWP as carrier.

Chapter 3:
Research question: are LMWPs catabolized in the kidney?
- what is the rate of renal catabolism?
- is there a difference in rate of catabolism between different LMWPs?

Application for drug targeting: the rate of renal drug release may be determined by the rate of LMWP catabolism in the kidney.

Chapter 4:
Research question: does proteinuria affect the renal handling of LMWPs?
- is the renal uptake of LMWPs hindered by proteinuria?
- is the renal catabolism of LMWPs affected by proteinuria?
- is the LMWP handling in specific segments of the proximal tubule affected by proteinuria?

Implication for drug targeting: during proteinuria the tubular system is exposed to an increased amount of protein. Drug-LMWP conjugate may have to compete with this overload of protein for tubular uptake and/or catabolism. Since not all proteins are taken up in the same segments of the proximal tubules, we may be able to deliver the drug specifically to the overloaded tubular cell during proteinuria or specifically circumvent the overloaded cells by choosing the right LMWP as a carrier.

Drug targeting
After characterization of the potential drug carriers, we focused on the in-vivo application involving drug targeting. Figure 3 together with table 2 summarize the two concepts studied in chapter 5 and 6 and 7. The first concept concerned drug targeting to the kidney (a). Naproxen was coupled via its carboxylic group to the free amino-groups of the LMWP lysozyme. Like native lysozyme, we expected the conjugate to be renal specific by glomerular filtration and reabsorption by the proximal tubular cell. During catabolism of the conjugate, the drug should be released from the conjugate, subsequently leave the lysosomes of the proximal tubular cell and reach the desired site of action. Naproxen is a NSAID, effective in the treatment of proteinuria [9] and tubular defects [10] by inhibition of prostaglandin synthesis. Little is known about the exact site of prostaglandin synthesis in the kidney that should be reached by the drug for a proper therapeutic effect. For instance, it is not known whether the glomerular prostaglandin synthesis should be blocked to reduce proteinuria or that an indirect effect due to a reduced tubular prostaglandin synthesis is adequate.

The second concept concerns bladder targeting (b). Doxorubicin was coupled to the LMWP lysozyme to deliver the drug specifically to the urinary tract. All 7 free amino-groups of lysozyme were shielded with doxorubicin molecules to prevent tubular uptake of the conjugate. In this way, doxorubicin targeting to the urinary bladder may be achieved. To allow release of the drug in acidified urine, an acid-labile spacer was intercalated between doxorubicin and lysozyme. Doxorubicin is an antitumor drug that, in its free form, is effective in the treatment of bladder carcinoma especially if administered via intravesical irrigation [3]. Thus, doxorubicin released from a suitable carrier that is excreted in the urinary bladder can in principle reach its site of action, the bladder carcinoma cells.

Figure 3

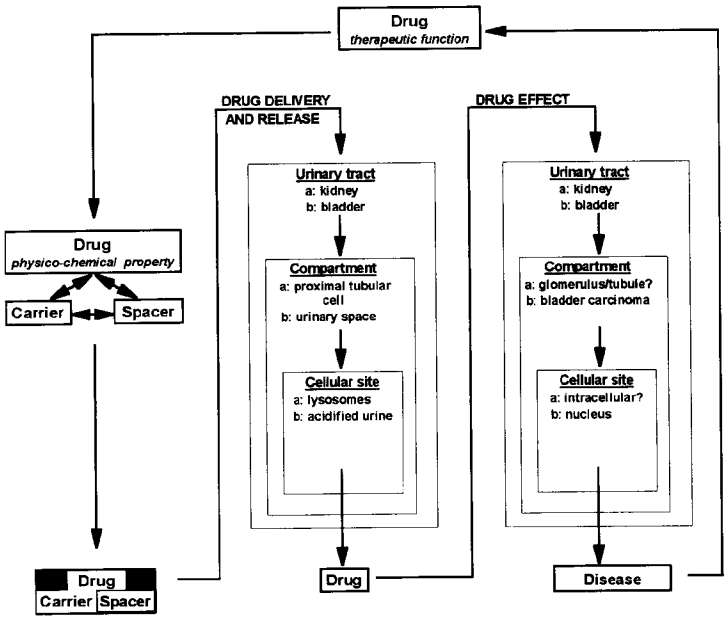

Table 2

Drug *Physico-chemical properties:* a: naproxen (carboxylic group) b: doxorubicin (amino-group)	Diseases a: proteinuria/tubular defects b: bladder carcinoma
Carriers a: LMWP (lysozyme) low coupling degree b: LMWP (lysozyme) high coupling degree	Drugs *Therapeutic function* a: inhibition of prostaglandin synthesis b: anti-tumor effect
Spacer a: none b: cis-aconityl (acid-labile)	

Chapter 5:
Research question: what are the kinetics of naproxen-lysozyme conjugate?
- is the conjugate predominantly taken up by the kidney?
- does conjugation to lysozyme increase the renal specificity of naproxen?
- is a conjugate of naproxen-lysozyme catabolized in the kidney with a release of the drug as result?

Chapter 6:
Research question: does the naproxen-lysozyme conjugate show renal effects?
- does it reduce renal prostaglandin synthesis?
- does it reduce the urinary excretion of sodium and water?

Chapter 7:
Research question: can doxorubicin be targeted to the urinary bladder using lysozyme as carrier?
- does conjugation to lysozyme increase the urinary excretion of doxorubicin?
- does conjugation to lysozyme prevent general and renal toxicity of doxorubicin?

Appendix
To study drug targeting, several experimental settings had to be optimized. Chapter 8 describes the newly developed automated set-up to collect rat urine during short-term drug intervention.

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Chapter 1

Renal drug targeting : optimalization of renal pharmaco-therapeutics

Marijke Haas, Dirk K.F. Meijer, Frits Moolenaar, Paul E. de Jong, Dick de Zeeuw

Yearbook of Nephrology 1996, Andreucci VE, Fine LG (eds), Oxford University Press, 1996, pp. 3-11.

Introduction

The first step in drug development is generally focused on the aspects of drug action. Specific enzymes or receptors related to the disease are identified and potential drugs are subsequently tested for their potency, affinity and selectivity. However, even if a compound is synthesized that has quite optimal pharmacodynamic characteristics, this does not necessarily imply that, therapeutically seen, an optimal drug is obtained. The pharmacokinetic properties should also be considered. For example, the drug may not be able to reach the site of action hindered by barriers. Furthermore, the localization of most target enzymes and receptors is not exclusive for the target tissue which may lead to undesirable side-effects. *Drug targeting* is the field of research that deals with these issues. It can be defined as the technology to direct a therapeutic agent specifically to the desired site of action with little or no interaction with non-target tissue. Although the idea of drug targeting was already proposed in 1906 by Ehrlich [1], progress in this field, especially to the clinical phase was only slow in the past decades [2-5].

The need for a drug targeting approach is evident in the case of extremely toxic agents that have to be administered in high doses such as antitumor drugs. But what would be the rational for specific drug targeting to the kidney? Clearly, the kidney is one of the organs with the highest exposure to drugs circulating in the body. Around 25% of the cardiac output flows through the two kidneys. In addition, many compounds are highly concentrated in the proximal tubule by active processes while high luminal concentration of drugs are reached due to water reabsorption. So, at first glance, one may conclude that little would be gained by renal drug targeting. However, there are several good reasons for renal-specific drug delivery. Firstly, although many drugs used in treatment of renal diseases do reach the kidney in sufficient amounts, they may cause serious extrarenal effects. Secondly, the renal transport of a drug may not be optimal in relation to the target cell within the organ. Thirdly, some drugs are largely inactivated before they reach the site of action. Finally, pathological conditions like abnormalities in glomerular filtration, tubular secretion, or the occurrence of proteinuria can affect the renal delivery of a drug. Renal-specific drug targeting is one option to overcome such problems and to improve the therapeutic index of a drug. Furthermore, cell-specific drug targeting within the kidney may provide an interesting tool to understand the mechanisms of drug action and to manipulate renal physiology. In the first part of this review we will discuss the principles of drug targeting in general and in the kidney specifically, while in the second part the pharmacological effects are reviewed.

Techniques of drug targeting

Because of the large variety of kinetic demands in the treatment of different diseases, there is not just one general approach to target a drug. Roughly, the approaches studied so far can be divided in three categories: local administration, the prodrug or softdrug approach and the targeting approach using macromolecular drug carriers.

Local administration is a rather simple way of targeting to topical sites [3]. Implanted infusion pumps and intra-arterial infusions can in principle be used for targeting to the kidney. However, these techniques are not generally applicable and their use is restricted to specific cases.

The technology of pro- and softdrugs involves one or more chemical modifications of the parent compound using chemical moieties that, with regard to size are comparable to or even smaller than the parent drug. Prodrugs are inactive derivatives of an active agent. Chemical transformation of the prodrug by, for instance site-specific enzymes can result in a site-specific activation of the drug. Especially of interest for topical applications are the so called "softdrugs". Softdrugs are active derivatives of inactive molecules. As soon as the softdrug is released at its target site, chemical deactivation prevents undesirable effects elsewhere in the body [4,5].

To mask the physicochemical properties of a drug, several kinds of carriers have been developed. Drugs have been incorporated in particles such as liposomes [6,7], albumin microspheres, cell ghosts, and (nano)-particles made out of synthetic polymers [2,3]. Particles have a high capacity of drug incorporation and drugs can easily be entrapped without the need of a chemical binding. However, due to their large size and to the fact that they are mainly phagocytosed by macrophages, their application is limited. Avoiding uptake of liposomes by the reticulo-endothelial system has been achieved by coating the liposomes with chains of polyethylene glycol, the so-called "stealth liposomes" [6,7]. By chemical synthesis, drugs have been coupled to soluble carrier proteins [8-11] and soluble polymers [12,13]. Macromolecular carriers are much smaller than the particle type of carrier and therefore applicable for targets not directly in contact with blood. Synthetic polymeric carriers can be tailor-made to predetermined specifications and possess a low or even absence of immunogenicity, while immunogenicity can be a serious drawback of protein carriers [12,13]. Site-specific delivery with drug carriers can be obtained either by using a carrier that is recognized selectively by the target cell (e.g. monoclonal antibodies, transferrin and certain glycoproteins) or by coating non-specific carriers (e.g. liposomes and polymers) with a site-specific ligand.

Before discussing which of these three options can be used for renal targeting, we will first review the potential targets in the kidney.

Renal "targets" for delivered drugs

Glomerulus

About 10% of the blood flow through the kidney is ultrafiltered in the glomerulus. Due to size- and charge-selective properties of the glomerular filter, drugs associated with large plasma proteins will be excluded from filtration (Figure 1) [14]. Modulating a drug in such a way that it is predominantly filtered by the kidney implicates that the drug will arrive in the tubular lumen and in principle may exert its activity in all compartments of the kidney downstream from the glomerulus.

The mesangium does not have a basement membrane and is therefore also perfused with macromolecules. Small compounds such as drugs pass through this compartment without being retained. On the other hand, entrapment of macromolecules, immune complexes and macrophages occurs, especially in the state of inflammation [15,16]. So, mesangial delivery of drugs might be achieved by associating the drug with certain macromolecules and blood cells.

Tubules

Different transport systems are involved in the secretion of various anionic and cationic drugs by the proximal tubular cell (Figure 1) [17]. Renal secretion is only possible for compounds of a size smaller than 1500 D. Therefore, an enhanced renal delivery via these processes can only be obtained by modulating the physicochemical properties of the drug with relatively small chemical entities and not with large carriers. An enhanced accumulation of drug in the proximal tubular cell may be important for drugs being active in this particular cell type while an accelerated tubular secretion may improve the therapeutic efficacy of agents that act at luminal and downstream sites.

Proteins and other polypeptides with a molecular weight of less than 60 kD (in healthy subjects) are filtered and reabsorbed by the proximal tubular cell. Particularly the low-molecular-weight proteins (LMWPs) with a molecular weight smaller than 20 kD are eliminated from the general circulation by this pathway due to an unrestricted glomerular filtration. In the cell, the ligand is entrapped in endosomes and subsequently migrates to the lysosomes (Figure 1). The lysosomes are instrumental in hydrolysis of these proteins: their pH is low (pH 4.5-5.5) and a large number of proteolytic enzymes is present [18]. In

principle it is possible to use this endocytosis system for renal delivery by coupling the drug to a LMWP or another polypeptide. After release of the drug within the lysosomes, the drug may exert its activity within the proximal tubular cell or may act after secretion in the lumen at downstream target sites.

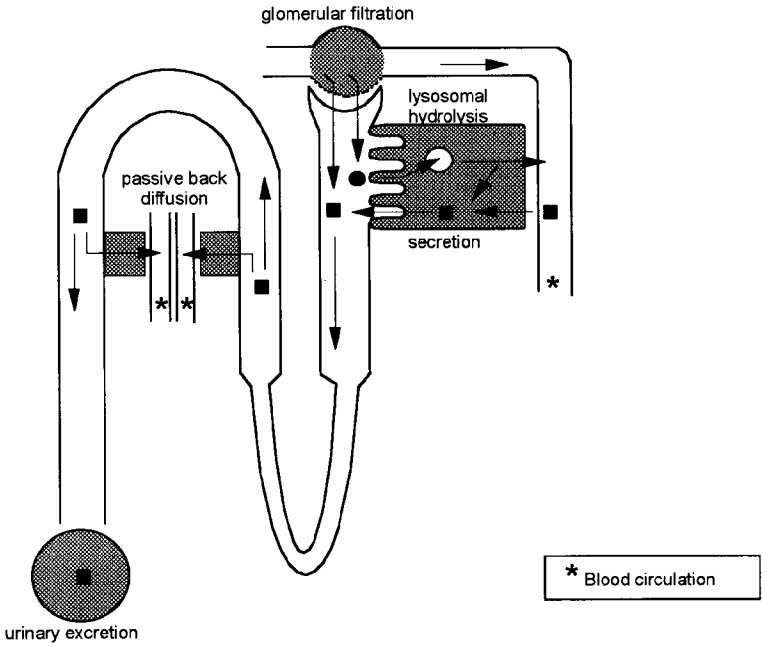


Figure 1. *Renal drug handling.* Drugs are filtered by the glomerulus when they are smaller than albumin and not associated with large plasma proteins. Low-molecular-weight proteins are filtered by the glomerulus and after proximal tubular reabsorption hydrolyzed in the lysosomes [●]. Several transport mechanisms exist for the secretion of drugs by the proximal tubular cells. Passive back diffusion into the blood circulation is only possible for drugs that are sufficiently lipophilic and not ionized in the urinary space.

Up to 99% of the filtered water is reabsorbed along the renal tubule. As a result, the concentration of filtered and/or secreted drug in the lumen increases considerably. This will stimulate passive diffusion into the tubular cells and the interstitium and eventually diffusion back to the blood circulation (Figure 1). Since only un-dissociated (non-ionized) drugs can pass the lipoid membrane of the tubular cells, the rate of reabsorption of the drug depends on the pH of the luminal fluid and the pK_a of dissociable groups as well as on the lipophilicity of the un-dissociated drug [14]. Theoretically, an enhanced interstitial

accumulation of a drug can be attained by intrarenal chemical conversion of the drug into an uncharged, lipophilic prodrug. An increase in total charge and hydrophilicity of the (pro)drug will generally result in an increased urinary excretion of the drug.

Renal enzymes
In the prodrug concept, site-specific enzymes are required to obtain the desirable site-specific release of the parent compound. Although enzymes are never entirely tissue specific, some rather renal selective enzymes have been identified and used for the design of renal-specific prodrugs. γ-Glutamyl transpeptidase is an cell-surface enzyme present at the brush-border of the proximal tubular cells and to a lesser extent at the basolateral membrane. l-Aminoacid decarboxylase, N-acetyl transferase, β-lyase and γ-glutamyl cyclotransferase are present inside the proximal tubular cell. In addition, the kidneys show a high activity of alkaline phosphatases. Drugs released extracellularly via enzymatic conversion will primarily be able to exert their effect in the lumen of the tubulus or downstream sites while intracellularly released drugs, at least initially, act upon intracellular sites.
Renal enzymes are also of use to uncouple a drug from a renal specific carrier. The brush-border of the proximal tubule is saturated with peptidases and esterases, whereas tubular lysosomes are loaded with endopeptidases, exopeptidases and esterases to degrade proteins [19,20]. The lysosomally released drug will be able to exert its activity intracellularly, initially in the lysosomes and subsequently in the cytosol after diffusion out of these organelles. After secretion from these cells, the drug may also act at distal sites in the urinary space and/or interstitium. Drugs released from the carrier already in the lumen may be active in the lumen and downstream.

From the previous section it is clear that there are many options for renal specific targeting. However, there are also many potential pitfalls that might corrupt the renal targeting strategy. In the following sections we will discuss the different strategies that have been examined up to now.

Potential procedures for drug targeting to the kidney

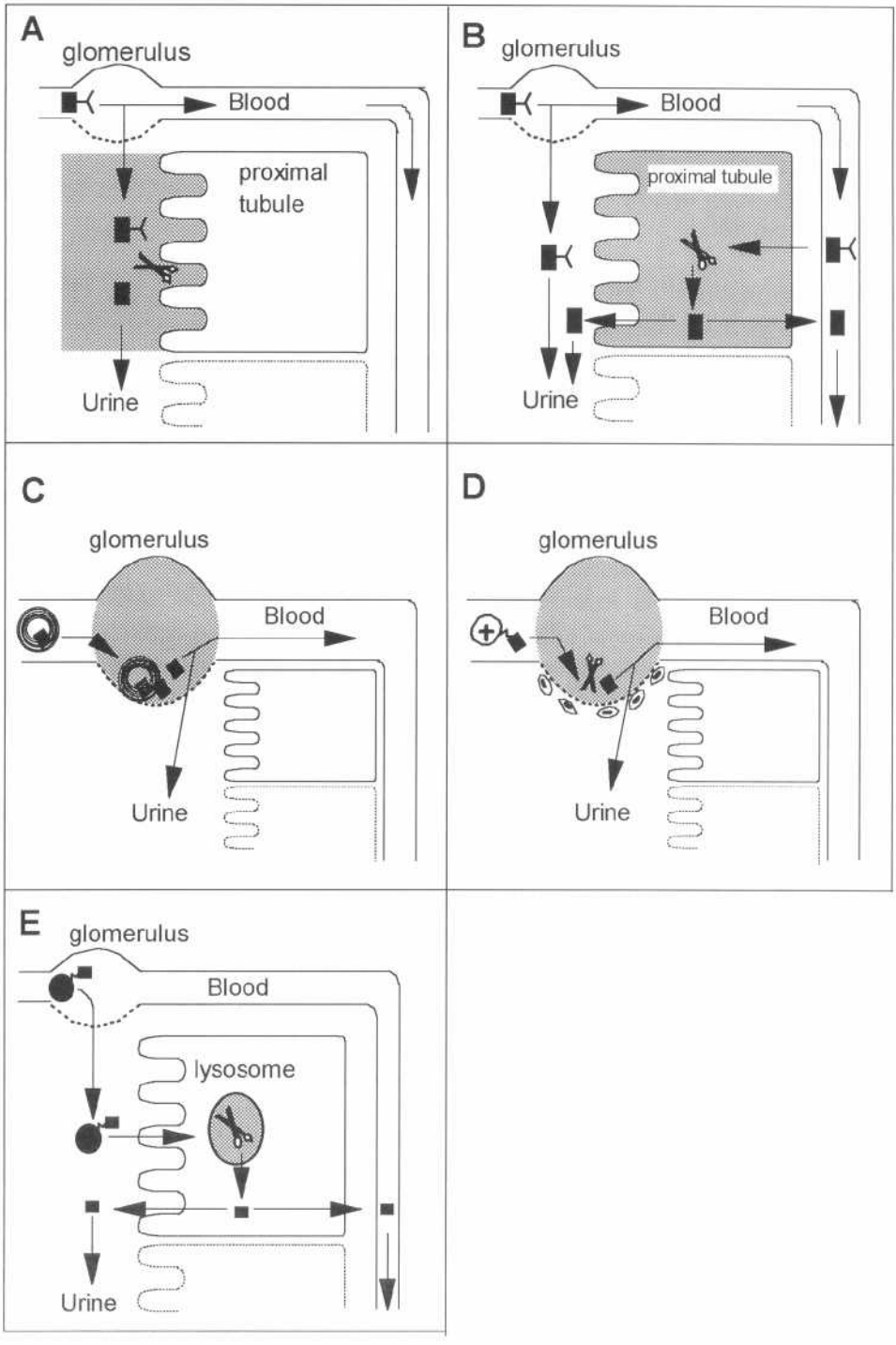
Local drug administration
A good but rather invasive example of local drug administration is the infusion of drugs into the renal artery. In a study of Ruers et al. [21], continuous infusion

of prednisolone in the renal artery of the rat resulted in a two times higher level of the drug in the kidney allograft compared to an intraperitoneal administration. The rather poor renal selectivity can be explained by the low renal extraction of prednisolone. Similarly invasive is the strategy of local irrigation of the bladder, e.g. with amphotericin B [22]. The benefits of such procedures have to be weighted against their potential problems for the individual patient.

Prodrugs/softdrugs

Several drugs have been derivatized with γ-glutamyl (glu), the substrate for γ-glutamyl transpeptidase. γ-Glutamyl transpeptidase is a renal-specific enzyme, predominantly located on the brush-border of the proximal tubule. Thus, γ-glutamyl prodrugs will be activated in the tubular lumen (Figure 2A). Both, l-dopa and dopamine have been coupled to γ-glutamyl. Gludopa [23-29] appeared to be a more renal effective prodrug than glu-dopamine [30]. Another example is the prodrug γ-glutamyl-sulfamethoxazole. This prodrug did not show a renal selectivity, either as the result of rapid removal from the kidney, or due to cleavage even in tissues containing only a low concentration of the enzyme. N-acetyl transferase is a renal specific enzyme located inside the proximal tubular cell. Therefore, N-acetylated drugs will be activated intracellularly (Figure 2B). In contrast to γ-glutamyl-sulfamethoxazole, N-acetyl-glutamyl derivatives of sulfamethoxazole showed a renal selective distribution [31,32]. N-acetyl-γ-glutamyl-aminowarfarin was not a successful prodrug since it appears not to be secreted via the tubule and thus does not reach the enzyme site [33]. Lastly, some successful prodrugs directed at renal ß-lyases and phosphatases have been reported [34,35].

Drugs that are released intracellularly, partly diffuse through the interstitium back to the blood circulation (Figure 2B). This might be an important pathway for reaching interstitial sites but also may imply an undesirable burden of the drug for extrarenal tissue after all. This last point was very elegantly solved by using a kind of softdrug/prodrug combination [36]. After administration of N-acetyl-glutamyl-CGP 18137, CGP 18137 was released in the cell and partly diffused back to the circulation. In the circulation this vasodilating agent is rapidly inactivated by a chemical reaction [37]. This example stresses the potential(s) of softdrugs in the field of drug targeting. Irrespective of the fact that the drug is generated from a prodrug or released from a carrier, inactivation of the active compound after being released into the blood circulation would be highly desirable.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glomerulus
Blood
Urine
E
glomerulus
Blood
lysosome
Urine

Figure 2. *Methods of renal drug targeting.*

A. Prodrugs may be substrates for brush-border enzymes resulting in release of the active drug in the tubular lumen and subsequent reabsorption at distal sites or elimination in the urine.

B. Prodrugs may be substrates for intracellular enzymes of the proximal tubular cell resulting in release of the active drug intracellularly and subsequent secretion into the tubular lumen or via the interstitium back into the circulation.

C. Particularly during inflammation, particle carriers are entrapped in the glomerulus. As a consequence, incorporated drugs are slowly released in this specific compartment.

D. Cationic macromolecular carriers bind to the negatively charged membranes of the glomerulus. Attached drugs can in principle be released by membrane enzymes or by intracellular enzymes after internalization in mesangial cells or macrophages.

E. Low-molecular-weight proteins are filtered by the glomerulus and after proximal tubular reabsorption hydrolyzed in the lysosomes. Thereafter, coupled drugs may be released in the lysosomes and subsequently secreted into the tubular lumen or back to the circulation.

The data show that renal-specific activation of a drug can be achieved by masking the active sites of the drug with a specific pro-moiety. Taking advantage of the localization of a renal specific enzyme, a drug can be activated either predominantly in the tubular lumen or within the proximal tubular cell. However, the design of such a drug formulation can be rather complicated. Ideally, the prodrug should not be cleaved extrarenally and after delivery to the kidney should be adequately exposed to the target enzyme. This can be obtained by choosing the pro-moiety not only on the basis of its enzyme affinity but also taking into account its effect on the renal transport of the prodrug. Lastly, if oral administration is desired, the ability to reach the general circulation intact from the gastro-intestinal tract should also be included in the design.

Particle type of carriers

Nanoparticles made of isobutylcyanoacrylate were tested for actinomycin B delivery to the mesangium of the glomerulus. After an intravenous injection of these nanoparticles the majority was removed by the reticulo-endothelial system. Still, a substantial amount was entrapped and phagocytosed in the mesangium. Interestingly, during inflammation the renal entrapment largely increased [38].

In a study of Mishina and Jusko [39], methylprednisolone was incorporated in liposomes with the intention to increase the drug level in the lymphatic tissue. Indeed, the spleen and thymus showed a significant increase of methylprednisolone after an intravenous injection in the rat. Interestingly, in the first couple of hours after administration the drug level in the kidney was four times higher than after administration of the parent drug.

In two studies, normal liposomes modified for gene-targeting were infused via a renal artery of a rat. Gene expression was only obtained in the perfused kidney. Neither the contralateral kidney nor other organs were transfected [40,41].

Kitamura et al. [42] introduced genes in cultured mesangial cells. Subsequently, the cells were infused via the renal artery of a rat. As with the intra-arterial liposomes, renal gene expression was found in the infused kidney whereas no gene expression could be detected elsewhere in the body.

The above mentioned data show the potential to target drugs specifically to the mesangium of the kidney by using particle carriers (Figure 2C), particularly in the inflamed state. Improvement of the mesangial entrapment might be obtained either by using "stealth liposomes" to reduce the uptake by the

reticulo-endothelial system, or by coating the particles with cationic groups or cell-specific antibody fragments.

Soluble macromolecules as drug carriers

As far as glomerular targeting is concerned, one could take advantage of the negative charge of the glomerular membranes by using cationic carriers (Figure 2D) [43,44]. Also mesangial uptake of macromolecules may be an interesting but as yet unexplored area.

Drug delivery to the tubule has been tested more extensively. In this respect, the low-molecular-weight protein (LMWP) is of interest as a drug carrier. We showed that the LMWPs are rapidly removed from the blood circulation and accumulate in the kidney to about 80% of the intravenously injected dose (Figure 3). Large differences were found between the rates of degradation of the various LMWPs [45]. The high renal uptake and varying rate of degradation make such LMWPs interesting tools for drug targeting to the proximal tubular cell. For chronic treatment a slowly degrading LMWP (e.g. aprotinin) might be preferred as drug carrier while for a short lasting, high dose treatment, a protein that is rapidly hydrolyzed (e.g. cytochrome-c) could be a more suitable choice.

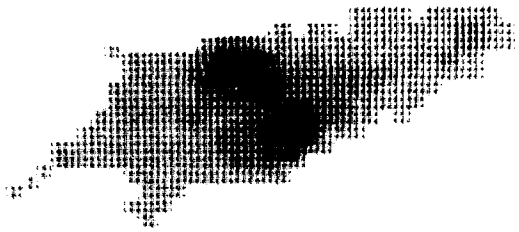

Figure 3. *Renal specificity of a radiolabeled LMWP.* Gamma-camera imaging after an intravenous injection of a radiolabeled low-molecular-weight protein (LMWP) in the rat, showing the predominant uptake of the LMWP by the kidneys.

The biotin-binding, truncated form of the LMWP streptavidin (14 kD) showed an extensive accumulation and a large resistance to/against hydrolysis in or at the tubuli (of the kidneys). The mechanism of renal accumulation has not yet been clarified. Especially if the mechanism of renal processing is not related to that of low-molecular-weight proteins but a result of interaction with cell-surface adhesion receptors, an interesting new potential drug carrier has been introduced [46]. Takakura et al. showed that blocking some of the aminogroups of the LMWP superoxide dismutase with mannose or galactosegroups resulted

in a reduced uptake in the proximal tubular cell and thus an enhanced exposure to the luminal surface [47].
In spite of the enormous variety of enzymes present in lysosomes, adequate uncoupling of a drug from a LMWP carrier can be a major problem. Direct coupling between the drug naproxen and the LMWP lysozyme via an amidebond did not result in the release of the parent drug in kidney homogenates [48]. Different spacer modalities between drugs and carriers, such as ester groups, specific poly-amino-acids [49-51] and acid-sensitive spacers [49,52,53] have been proposed to obtain a sufficient release rate for the drug. Indeed, introduction of an ester spacer (L-lactic acid) between naproxen and lysozyme resulted in a parent drug release in kidney homogenates [54]. Taking advantage of the acid environment in the lysosomes, the introduction of an acid-sensitive spacer between the drug sulfamethoxazole and lysozyme resulted in a conjugate that was stable in kidney homogenates at pH 7.5 but cleavable at pH 5.0 [55].
The data show that renal specific delivery and release of a drug is possible by coupling the drug to a low-molecular-weight protein (Figure 2E). Yet, a well chosen spacer is required for a programmed release of the drug adjusted to the therapeutic effects aimed at.

Polymers as drug carriers
Poly-aspartic acid with an average molecular weight of 15.000 D accumulates in the proximal tubular cells [56]. We observed that poly-lysine molecules larger than about 10.000 D are restricted from filtration whereas molecules smaller than about 3.000 D were rapidly excreted into the urine. Molecules with an intermediate size are filtered but effectively reabsorbed by the proximal tubular cells. Therefore, large cationic polymers might be useful for glomerular entrapment, small polymers for delivery to the urinary tract while the intermediate polymers could be used for the delivery of drugs to tubular cells.

Monoclonal antibodies for cell recognition
As far as we know, no renal specific drug-antibody constructs have been described. However, the potentials of such highly cell-specific compounds must be considerable. Except for using the intact antibody as a drug carrier, antibody fragments can be coupled to the drug or drug carrier for delivery to the antigen with high specificity [57].

Pharmacological effects of renal drug delivery systems

Removal of glomerular immune complexes

In many forms of glomerulonephritis glomerular immune complexes are present. Glomerular delivery of proteases that dissolve deposited immune complexes would be an interesting therapeutic approach [43]. The cationic protein avidin A was injected intravenously in rats with active serum sickness nephritis. Avidin was shown to be absorbed on the negatively charged membrane of the glomerulus. Since avidin has a high binding affinity for biotin, it was expected that subsequently injected biotinylated proteases would be targeted to the glomerulus. Indeed, treatment with biotinylated proteases showed an enhanced deposition of proteases in the glomerulus. This resulted in a more pronounced decrease in glomerular deposition of immune complexes and consequently a greater reduction of proteinuria than after administration of free proteases. However, the required parenteral administration of the modified avidin and biotinylated proteases limits the clinical application to short term treatment. This promising targeting approach has not been tested in the clinic yet.

Renal immunosuppression

Immunosuppression is widely used in different renal diseases as well as in kidney transplantation. However, since immunosuppressive agents show serious systemic side-effects, local drug delivery could be helpful. Ruers et al. [21] studied the impact of a locally administered immunosuppressive on rat allograft rejection. Prednisolone was infused through the renal artery and the different phases of allograft rejection were examined. During local prednisolone infusion, the early infiltration of activated white cells and macrophages was not prevented which indicates that systemic responses were not inhibited. However, T-cells and macrophages in the allograft did not destroy the tissue. This could be explained by the successful inhibition of local IL-2-R expression, INF-γ excretion and MHC class II induction. Although the method itself is not applicable for chronic treatment, this study clearly shows that renal specific targeting of immunosuppressives is effective.

Mishina and Jusko [39] showed in rats that after administration in liposomes, the renal methylprednisolone concentration was fourfold higher compared to dosing with the parent drug. However, unfortunately no effect was analyzed.

Treatment of reperfusion ischemia

Members of the superoxide family are suggested to be responsible for tissue damage, especially during reperfusion. The therapeutic effect of superoxide dismutase (SOD) and three of its derivatives on acute renal failure was studied in rats [44]. Cationized SOD and a long circulating SOD derivate (PEG-SOD) showed the ability to protect the kidney from ischemic injury at a dose at which native SOD was not effective. The potential for clinical use is high. The parenteral administration can be used here since only an acute protection against reperfusion ischemia during kidney transplantation or after acute renal failure will be the aim of the treatment. To date no clinical studies have been performed.

Influencing renal hemodynamics and treatment of proteinuria

Many non-steroidal anti-inflammatory drugs have a renal therapeutic target in reducing urinary protein excretion in proteinuric patients [58]. Since this application of NSAIDs is limited due to the rather high dose required and the serious side-effects on the gastro-intestinal tract [59] and central nervous system, renal specific drug delivery might be useful. Franssen at al. [48] coupled the long-acting NSAID naproxen to the LMWP lysozyme. This conjugate showed predominant renal uptake such that the renal concentration of naproxen was 70 times higher compared to uncoupled naproxen after intravenous injection in the rat [60]. The drug was slowly released from the kidney [60] probably as naproxen-lysine [48]. This metabolite was, like native naproxen capable of inhibiting the *in vitro* synthesis of prostaglandin E_2 . In a second study, the parent naproxen was released by coupling naproxen to lysozyme via an l-lactic acid spacer [54]. Although this approach appears to be promising, *in vivo* effects on proteinuria are yet to be tested. If indeed effective, the targeted naproxen may be a powerful tool for the treatment of proteinuric patients in spite of the need for parenteral administration. None of the alternative strategies studied so far showed such an improved renal delivery and potential to control the rate of renal drug release.

Renal selective delivery of drugs interfering in the renin-angiotensin system may be valuable for two reasons. First, the renal effects [61] may then occur without systemic effects. Second, a renal-specific delivery could be attractive since the renal renin-angiotensin system may be primarily responsible for certain renal and hypertension related diseases [62,63]. We recently coupled the angiotensin-converting enzyme inhibitors captopril to the LMWP lysozyme [64] and are now investigating the renal kinetics and renal effects of the targeted drug.

Renal specific delivery may improve the therapeutic index of dopamine for the treatment of essential hypertension or the prevention of renal ischemia [65,66]. One example of such a renal specific compound is γ-glutamyl-l-dopa (gludopa). Gludopa has been studied extensively in animals and humans. Wilk et al. [23] found a five times higher renal accumulation of dopamine after prodrug than after free dopamine injection in mice. Most of the investigators agree that the prodrug is mainly active in the kidneys [23-28]. It increases the renal blood flow and water, salt excretion and reduces the plasma renin activity. Only at doses 20 times higher than those necessary for the renal effects [23] and after a prolonged infusion [28] systemic effects like blood pressure reduction were found. Unfortunately its clinical use is limited by the poor oral bioavailability (1 to 2%).

A rather new (1988) renal-selective prodrug is the epinine-4-O-phosphate (SIM 2055). Specifically in the kidney, SIM 2055 is converted by phosphatases into epinine, a dopaminergic agonist 5 times more potent than dopamine itself. In anesthetized dogs, the drug showed an increase in renal blood flow and decrease in renal vascular resistance with no detectable systemic changes in blood pressure, left ventricular pressure and heart rate. The oral bioavailability was high (70%) [35]. Thus, this prodrug was expected to have better clinical perspectives than gludopa. However, for reasons unknown to us this product was not further developed.

We are still attempting to couple dopamine to LMWPs in order to deliver the compound to tubular cells via the earlier mentioned endocytosis process. However, no firm conclusions can be drawn from the present data, since the chemical synthesis of the preparation is cumbersome and the renal effects on sodium excretion and urine production highly variable.

CGP 22979 is the N-acetyl-glu prodrug of the hydralazine-like vasodilator CGP 18137. In the rat the prodrug showed a concentration-dependent decrease in renal resistance without an effect on blood pressure. In contrast, the parent drug CGP 18137 showed an effect on both renal resistance and blood pressure [37]. Although the drug has the ideal kinetic properties to serve as a renal-specific vasodilator, for reasons unknown to us this product was not further developed.

Treatment of urinary tract infection

The rationale for renal targeting of sulfamethoxazole is its low extraction by the kidneys. Orlowski et al. [31] and Drieman et al. [32] studied the mechanism of renal handling of several derivatives of sulfamethoxazole *in-vitro* and *in-vivo*. The intracellularly released N-chloroacetyl-glu-sulfamethoxazole appeared to

be the most renal selective prodrug tested. The concentration of sulfamethoxazole in mice kidneys 20 min after intra-peritoneal administration was 2.2 times higher after N-chloroacetyl-glu-sulfamethoxazole than after an equimolar dose of free sulfamethoxazole [31]. Further studies with the compound have not been reported.

Franssen et al. [55] studied the sulfamethoxazole-aconityl acid-lysozyme conjugate. The major part of intravenously injected free sulfamethoxazole was excreted into the urine as the hepatic metabolite N-acetyl-sulfametoxazole. After injection of the conjugate the majority of the sulfamethoxazole was eliminated in the urine unchanged or in the free form while no hepatic metabolites could be detected. These data clearly indicate renal specific delivery of sulfamethoxazole by coupling the drug to a LMWP with an acid-sensitive spacer. To date, the clinical relevance of such a construct is rather low since very effective orally applicable alternatives are available for the treatment of uncomplicated urinary tract infections. The study indeed was performed for conceptual reasons rather than for clinical practice.

Aggressive, intravenous therapy is often required to reduce the urinary tract infection in circumstances such as fungal infections during immunosuppressive therapy [67]. For this purpose the antibiotic drug amphotericin B is often used in spite of its severe toxicity and low renal extraction. Urinary tract infection caused by *Candida albicans* in humans was treated successfully by irrigating the bladder with amphotericin B [22]. Although effective in many patients, bladder irrigation is not indicated in complicated or upper tract infection and recolonization is common after discontinuation of the treatment.

Incorporating amphotericin B into liposomes has shown considerable promise as an alternative for the treatment of systemic fungal infection with intravenous amphotericin B/deoxycholate suspension (Fungizone®). Ralph et al. [67] studied the effect of liposomal amphotericin B in 4 hospitalized patients with complicated urinary tract infection. In all patients, the preparation was successful in the treatment of the urinary infection while being well tolerated. The two renal transplant patients showed a small but reversible rise in creatinine after the treatment with liposomal amphotericin B, indicating only a mild renal toxicity of this formulation. Taken into account that liposomes are rapidly removed by the reticulo-endothelial system and are too large to be filtered in the glomeruli, the authors called this a rather surprising result. The only plausible explanation we can think of is an entrapment of the liposomes in the glomeruli leading to a slow release of the drug in the tubular lumen. Because the patients in question are mostly hospitalized, parenteral administration is not a major limitation.

An intriguing future approach for the treatment of urinary tract infection is the coupling of antibiotics to small sized molecules using an acid-sensitive spacer. Such conjugates should be designed in such a way that they are freely filtered and/or secreted into the tubular lumen. By inducing a urine with a low pH, the parent compound will be released locally.

Targeting for diagnostic purpose

The strategies used to deliver drugs can obviously also be used to deliver radio-isotopes to the kidney. Indeed, Bianchi et al. labeled the LMWP aprotinin with ^{99m}Technetium to measure proximal tubular function by external counting [68]. This method was further improved by Rustom et al. [69]. We have recently tested a concept to improve the rubidium/krypton technique for measuring renal blood flow. A rubidium complexing agent (calixspherand) was coupled to the LMWP lysozyme to obtain renal-specific delivery [70]. *In-vivo* studies (unpublished observation) showed that a higher hydrophilicity of the rubidium complexing agent is required to prevent distribution to the reticulo-endothelial system.

Targeting of genes to the kidney

Gene targeting stands for the cell specific introduction of exogenous genes. The potential application lies in the correction of genetic diseases. However, it may also be of interest to use this approach in the treatment of chronic renal diseases. Several groups recently succeeded in introducing genes in the glomerulus of the rat kidney. In two studies [40,41], the particular gene was introduced using liposomes as vehicles. Tomita et al. [40] showed that gene expression occurred in the perfused kidney and that this was transient (several days). This transient expression may offer the advantage of a low risk of excessive gene expression. However, the present time span of expression should be further expanded to make gene therapy an attractive intervention. Isaka et al. [41] introduced the genes for transforming growth factor-ß (TGF-ß) and platelet-derived growth factor (PDGF) into the glomerulus. It has been suggested that these factors contribute to the development of glomerulosclerosis. Indeed, after the overexpression in the glomeruli, rapid glomerulosclerosis developed.

Kitamura et al. [42] introduced a reporter gene which encodes bacterial ß-galactosidase in the rat kidney by injection *in-vitro* transfected mesangial cells via the renal artery. To amplify the in situ expression, the transfected cells were introduced in glomeruli subjected to transient mesangiolysis by a specific

monoclonal antibody. Compared to the liposome method, this system achieved a higher efficiency and a longer time span of expression (4 to 8 weeks).
Both, the liposome and mesangial cell vector system do not appear to have clinical future in the native kidney. However, such systems may be of use in the transplant kidney, such as with the introduction of specific genes that regulate immune responses.

Perspectives in renal targeting technology

Viewing the present state of the art of renal drug targeting one may say that many opportunities are offered. Yet most of the procedures proposed so far are still in the preclinical exploration phase. The largest progress was probably made in the development of renal prodrugs. This is not surprising considering the versatile experience that could be adopted from the research fields of bioavailability and brain delivery [71]. Although the modification to prodrugs is appealing, there are still several limitations with regard to cell-specificity. We anticipate that the macromolecular targeting approach may provide solutions to overcome some of these problems.
The first that should be proven before drug targeting will be accepted as an important method to improve the therapy of renal diseases is not the demonstration of an increased drug delivery to the kidney but more importantly, a markedly increased therapeutic effect. Only very few, if any, compounds have reached that state of research. The lack of such convincing proof hinders rapid development of effective new, renal-specific drug formulations. Eventually, drug targeting concepts should be included in the process of drug design and development in an early stage and should be less an art of problem solving in retrospect.

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Chapter 2

Quantification of renal low-molecular-weight protein handling in the intact rat

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Introduction

In the kidney, low-molecular-weight proteins (LMWPs) are relatively freely filtered and after reabsorption by the proximal tubular cells, intracellularly catabolized into small peptides and single amino-acids [1]. As such, the kidney plays an important role in the elimination of circulating LMWPs. Studies aimed at quantifying the renal contribution to catabolism of LMWPs revealed rather variable results. On the one hand a major, if not exclusive, renal contribution (60-90%) to the total clearance was claimed on the basis of the renal extraction measurements of the particular LMWP [2-4]. On the other hand, several studies showed a considerable lower renal contribution (30-60%), calculated on the basis of the actual amount of administered LMWP measured in the kidneys [6-10]. The question is whether methodological differences can explain the observed discrepancies.
With regard to the renal rate of degradation, large differences have been found for individual LMWPs. Previous studies on this topic were performed either *in vitro* kidney preparations [2,3,7,11], or by measuring the renal LMWP concentration in extirpated kidneys at different time intervals [7,8]. The disadvantage of the latter *in vivo* studies is the low time resolution. Bianchi et al. introduced an elegant non-invasive technique with a high time resolution with which an indication of the renal degradation of a LMWP was obtained [9].
In the present study we studied the renal handling of the three LMWPs cytochrome-c (Cy), lysozyme (Ly) and aprotinin (Ap), chosen on the basis of their alleged difference in renal half life [9]. We used a technique based on that previously reported by Bianchi et al. in which the renal handling of a radiolabeled LMWP was monitored by external counting in the intact rat [9]. We modified this technique to enable quantification of both renal uptake and degradation of a ^{123}I-labeled LMWP. To validate the renal uptake data obtained with the ^{123}I-LMWP, we used a second label, ^{131}I-tyramine-cellobiose (TC), a radiolabel that is retained within the cell in which it is internalized.

Materials and methods

Materials
Cytochrome-c (horse heart; MW 12,400; pI 10.6), lysozyme (egg-white; MW 14,300; pI 11) and aprotinin (bovine lung; MW 6,500; pI 10.5) were obtained from Sigma (Axel, The Netherlands).

Experimental set-up

One week before the experiments, female Wistar rats were instrumented with a permanent venous heart cannula [12]. The day before the experiment, the animals were fasted overnight and received 2 mg sodium-iodine intravenously to minimize the accumulation of unbound iodine in the thyroid glands and stomach [13]. Anesthesia was induced with halothane and a single dose of 30 mg/kg pentobarbital. During the experiment, anesthesia was maintained by continuous infusion of 4 mg/kg/h pentobarbital. In addition, the rats received a continuous infusion of 5% glucose (2.0 ml/h) to induce a stable urine production and 6 mg/h 3-monoiodo-l-tyrosine (MIT) to saturate the deiodinases [14]. Three rats were properly fixed on a middle-energy collimator of a gamma-camera. A window was selected on the peak energy of ^{123}I and ^{131}I with a width of 150 and 250 keV, respectively. Body temperature was monitored and maintained on 38°C with a heating pad and a lamp. Urine was collected continuously via a short cannula in the urinary bladder, draining into a tube situated on the collimator. The urine tube was exchanged after a half or one hour collection time. After a stabilization of one hour, 1.1 MBq ^{123}I-LMWP was co-injected with 0.55 MBq ^{131}I-TC-LMWP intravenously. The gamma-camera recorded the activity of both iodine isotopes in one or five minute time frames for 3 to 8 hours, depending on the LMWP studied. The radioactivity time-course of the right kidney, urine and total body was subsequently plotted after analysis of the respective "regions of interest". The amount of radioactive iodine within the total body in the first five minutes after injection was assumed to be 100% of the injected dose. Blood and urine samples were taken to measure the ratio of protein bound, amino-acid bound or unbound radioactivity. For each studied LMWP six rats were analyzed.

^{123}I-LMWP synthesis

^{123}I-LMWP labeling was performed according to the chloramine-T method of Hunter [15]. In short, 50 ml protein (10 mg/ml) and 10 ml chloramine-T (5 mg/ml) were added to 37 MBq Na^{123}I in 100 ml phosphate buffer (0.2M, pH 7.4) and mixed for 60 seconds. The reaction was stopped by addition of 25 ml metabisulfite (2.5 mg/ml). Free iodine was removed by a separation on a Sephadex G25 column. The protein fraction was used within two hours. Just before administration, 95% (Cy), 95% (Ly) and 94% (Ap) of the iodine was protein bound.

^{131}I-TC-LMWP synthesis

Synthesis of tyramine-cellobiose (TC) was performed by the reductive amination of cellobiose with tyramine [16,17]. In short, 10 mmol cellobiose, 10 mmol tyramine hydrochloride and 10 mmol propionic acid were dissolved in 40 ml methanol.

Sodium cyanoborohydride (12 mmol) was dissolved in 15 ml methanol and added drop-wise. The mixture was refluxed overnight. Acetone (300 ml) was added and the precipitate was filtered. The precipitate was dissolved in water overnight and applied to a cation exchange column (Dowex W50-X4; size 0.25x20 cm). The column was eluted with 0.5 M ammonia and the absorbance measured at 279 nm. The first peak was collected and lyophilized twice in order to remove traces of ammonia. The yield was about 40%.

^{131}I-TC labeling and subsequently coupling to LMWP was also carried out according to the method of Hysing [16], the modified version of Pittman [17]. In short, in an iodogen-coated tube, 10 ml tyramine-cellobiose (TC) (0.01M) in phosphate buffer (0.02M, pH 7.2) and 92 MBq Na^{131}I were incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 10 ml sodiumsulfite (0.05M) and 5 ml potassium-iodine (0.1M). The solution was transferred to a clean tube to couple the iodinated TC to the LMWP. Cyanuric chloride (30 nmol) in 20 ml acetone and 5 ml NaOH (0.01M) were added. After mixing for 30 seconds, 10 ml LMWP (100 mg/ml) in carbonate buffer (0.01M, pH 9) was added and the ^{131}I-TC-LMWP complex was separated from free compounds on a Sephadex G25 column. The protein fraction appeared to be very stable and was used within 40 hours. Just before administration, 100% (Cy), 100% (Ly) and 99% (Ap) of the iodine was protein bound.

Blood and urine analysis

The nature of the radioactive compounds in blood and urine samples was determined according to the method of Albert [18], slightly modified for analyzing small samples. In short, the LMWP coupled radiolabel in urine was measured by a trichloroacetic acid (TCA) precipitation of the LMWP. Urine (100 ml) and 400 ml 0.5% bovine serum albumin (BSA) were added to 250 ml TCA (15% TCA, 0.1% NaI, 0.1% tyrosine). After a high speed centrifugation of one minute the precipitate was washed with 200 ml 1M NaOH, to remove co-precipitated free iodine and reprecipitated with 300 ml TCA. After centrifugation, the radioactivity in the precipitate represented the LMWP coupled iodine. The TCA soluble radioactivity was subsequently analyzed for amino-acid coupled and free iodine. The TCA supernatant was diluted with ± 50 ml 32% NaOH and congo-red, to change the solution to a basic environment. Addition of 10 ml 50% HNO_3 and subsequently 250 ml 10% $AgNO_3$ in 2% HNO_3, was performed for the crystallization of the free iodine with silver. After centrifugation, the crystals were discarded to prevent interference with the counting of radioactivity. The radioactivity in the supernatant represents the amino-acid bound iodine. The unbound iodine could be calculated from the difference between radioactivity in the untreated urine sample and the

protein plus amino-acid fractions. The procedure for plasma samples was the same except for the first step: 10 ml plasma and 400 ml 0.35% BSA were added to 250 ml TCA. Radiolabeled aprotinin appeared to be TCA-precipitable for only 50%. The rest was recovered in the unbound iodine fraction after AgI crystallization. The administered cytochrome-c and lysozyme were analyzed likewise. The protein fraction of the administered aprotinin was analyzed by thin layer chromatography.

Quantification of the gamma-camera data

The data on radioactivity of the ^{123}I and ^{131}I measured over the different regions in the rat by external counting had to be transformed to the actual amount of label within the organ. To enable this, we studied the following biasing parameters: 1) the degree of scattering of the gamma-emittance by surrounding tissue, 2) the spillover of ^{131}I pulses into the ^{123}I channel 3) the background. The relative contribution of these factors was measured in a phantom rat model with the dimensions similar to the *in vivo* situation. A plastic tank (19x9x2 cm^3), representing the total body of the rat, held two artificial plastic kidneys (1.3 cm^3x 0.5 cm) and a urine tube of 1 ml, the average volume present in the *in vivo* experiment. The different compartments were filled with saline and various concentrations of ^{123}I or ^{131}I. Different sizes of the regions of interest were analyzed for the iodine radioactivity. This resulted in the following corrections for the *in vivo* experiments. For the ^{123}I, the region of the total body and urine tube contained the same amount of radioactivity compared to the total field of the camera. To estimate the total amount of ^{123}I-radioactivity present in the right kidney, a region of interest was needed with a size twice as large as the actual kidney. No corrections were needed for the background or the scattering effect of the radioactivity present in the left kidney. The counting efficiency of the ^{131}I appeared to be much lower. The total body and urine tube region contained 80%, whereas the 2 cm^2 sized right kidney region only comprised 47% of the total field radioactivity. Furthermore, a correction was made for the spillover of ^{131}I pulses into the ^{123}I channel. This spillover appeared to be 35% for the kidney and 50% for the total body and urine tube. No corrections were needed for background or scattering. To ensure that indeed no background corrections are needed, we studied the effect of an estimated background on the renal time-activity curve of ^{123}I-cytochrome-c (the LMWP that will be effected mostly). This process did not have a major impact on the results since the rates of degradation of the ^{123}I-cytochrome-c were not changed significantly. To validate whether the corrections of the ^{131}I gamma-camera data are justified in the *in vivo* studies with the combination of both radiolabels, we compared the renal uptake of ^{131}I-TC-lysozyme and ^{123}I-TC-lysozyme in two separate *in vivo* experiments. The renal

uptake appeared to be similar which proves that the ^{131}I corrections estimated from the phantom model experiment are indeed valid for the *in vivo* situation.

Pharmacokinetic and statistical analysis

The pharmacokinetic analysis of the plasma and kidney time-activity curves was performed using a computer program for non-linear curve-fitting, MultiFit (Department of Pharmacology and Therapeutics, University of Groningen), using the simplex algorithm [19]. Initial parameter estimates are automatically obtained by a curve-stripping procedure. The reciprocal of the y-value predicted by the model was used as the weighing factor [20].

The distribution volume of the LMWP is estimated from its plasma elimination.

All data are expressed as mean ± SEM. Statistical analysis was performed with the Wilcoxon rank test.

Results

Plasma disappearance of the radiolabeled LMWPs

After the intravenous injection of the ^{123}I and ^{131}I-TC radiolabeled LMWP's cytochrome-c, lysozyme and aprotinin, the plasma disappearance was determined by measuring the TCA precipitable plasma radioactivity in time (panels A figure 1). The results show that for each LMWP a similar biphasic plasma disappearance pattern was found for the two labels, ^{123}I and ^{131}I-TC (table 1). Apparently, the difference in the two labels did not affect the plasma elimination of the LMWP. Interestingly, compared to one another, the estimated distribution volume of the three ^{131}I-TC-LMWPs is markedly different: 11 ml for cytochrome-c, 20 ml for lysozyme and 50 ml for aprotinin.

		PLASMA HALF-LIFE (min)		
LMWP	phase(%)	^{123}I-LMWP	^{131}I-TC-LMWP	
cytochrome-c	1st (94)	2.1, 2.4	2.4, 2.8	(n = 2)
	2nd (6)	133, 167	90, 127	
lysozyme	1st (94)	2.6 ± 0.3	3.0 ± 0.2	(n = 4)
	2nd (6)	180 ± 23	259 ± 17	
aprotinin	1st (96)	2.0 ± 0.2	1.9 ± 0.2	(n = 5)
	2nd (4)	657 ± 62	646 ± 126	

Table 1. *Plasma disappearance of radiolabeled LMWPs after intravenous injection in the rat.* Data are given as mean ± SEM accept for cytochrome-c where results of both experiments are separately given. The values in parenthesis denote the contribution of the two phases relative to the total plasma disappearance. The first phase represents the distribution and first pass elimination of the LMWP from the plasma, the second phase the plasma elimination.

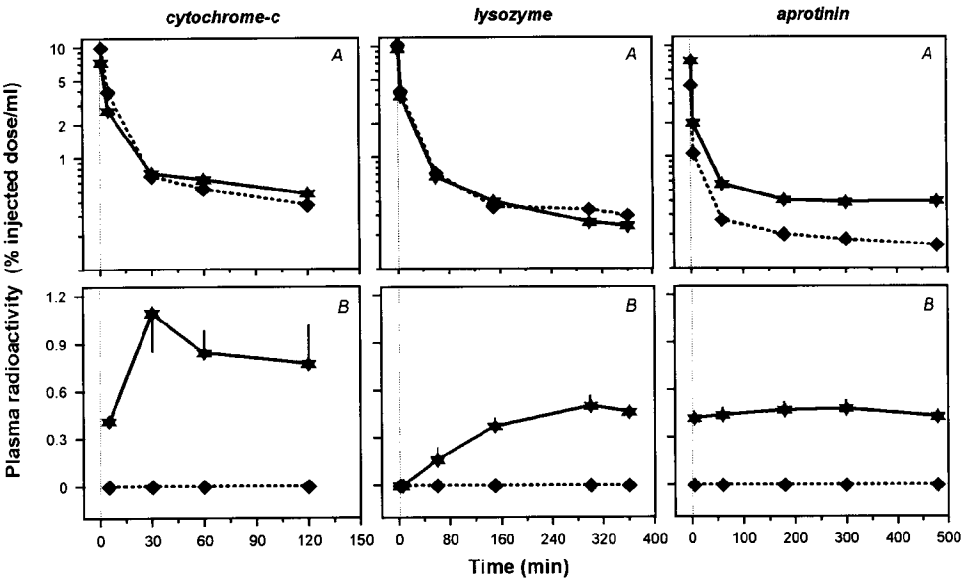

Figure 1. *Plasma radioactivity as a function of time after the intravenous injection of ^{123}I and ^{131}I-TC radiolabeled cytochrome-c (left panels), lysozyme (middle panels) and aprotinin (right panels) in the rat.. Panels A:* plasma disappearance of ^{123}I-LMWPs (solid line) and ^{131}I-TC-LMWPs (dotted line), measured as TCA precipitable radioactivity. *Panels B:* plasma appearance of the ^{123}I-degradation products (solid line) and ^{131}I-TC-degradation products (dotted line) as the TCA soluble radioactivity. Values are given as mean + SEM [Note: the SEM of the plasma concentration LMWP (panels A) is not visible since the SEM falls within the size of the marker].

Renal ^{123}I time-course

The body distribution of the radiolabel ^{123}I was monitored continuously by external counting. Following the intravenous injection, a large amount of the ^{123}I-LMWP is directly taken up by the kidney. After this first pass uptake, the renal uptake continues gradually in combination with a degradation of the LMWP in the kidney. Kinetic data of renal uptake and degradation were obtained by computer-aided kinetic analysis. Figure 2 shows the renal time-course of the three ^{123}I radiolabeled LMWPs cytochrome-c, lysozyme and aprotinin. Furthermore, the regression lines are drawn which represent the renal rate of uptake (a) and degradation (b). The results (table 2) show that the rate of renal uptake as well as degradation is very different for the three LMWPs. Lysozyme is taken up and degraded twice as fast as aprotinin. Cytochrome-c's rate of uptake is 8 times higher while the majority degrades 40 times faster than aprotinin. To verify that ^{123}I degradation products were not retained within the kidney, thus obscuring our degradation analyses, we measured the amount of ^{123}I bound to lysozyme in extirpated kidneys. At the end

of the experiment, 84 ± 2% (n=3) of the ^{123}I in the kidney appeared to be bound to lysozyme. This is in agreement with other studies [1,6] which show that at least 85% of renal radioactivity is protein bound, independent of the analysis time after injection.

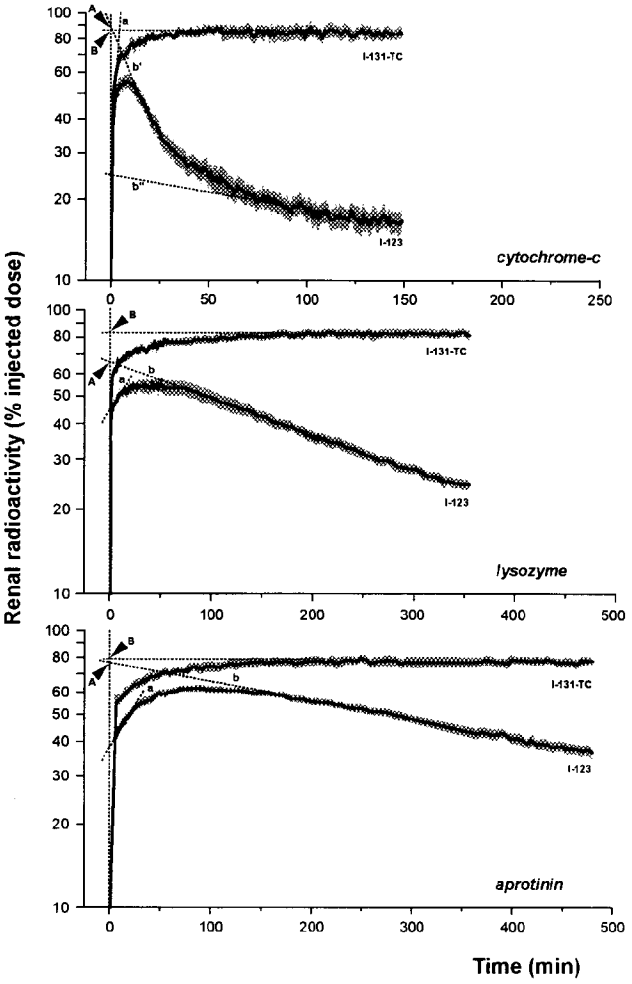

Figure 2. *Renal time-course of ^{123}I and ^{131}I-TC radiolabeled cytochrome-c (upper panel), lysozyme (middle panel) and aprotinin (lower panel) after intravenous injection in 6 rats.* Values are given as mean ± SEM (shaded area). Regression lines (obtained by kinetic analyses) are drawn, representing the rate of renal uptake (a) and renal degradation (b) of the ^{123}I-LMWP. The degradation of cytochrome-c appeared to be biphasic (b' and b"). Total amount of renal uptake is presented by the extrapolated degradation curve of ^{123}I-LMWP to the time of injection (A) and by the maximum level of radioactivity of ^{131}I-TC-LMWP (B).

	RENAL HALF-LIFE (t½ in min)		
LMWP	uptake		degradation
cytochrome-c	4.0 ± 0.1	1st	11 ± 1 (83 ± 6%)
		2nd	222 ± 46 (17 ± 1%)
lysozyme	14.2 ± 1.3		252 ± 26 (100%)
aprotinin	31.2 ± 0.9		443 ± 28 (100%)

Table 2. *Renal kinetics of ^{123}I-LMWP after intravenous injection in 6 rats.* Data are given as mean ± SEM. The t½ uptake is defined as the time in which the renal content, after the first pass uptake, increases with 50%. The t½ degradation is the time in which 50% is released from the kidney. Numbers in parenthesis denote the contribution of the different degradation phases relative to the total renal degradation of the LMWP.

Renal ^{131}I time-course

The renal time-course of the ^{131}I-TC-LMWPs was also recorded by external counting. After the first pass uptake, the renal uptake continues gradually until a plateau level is reached (figure 2). This plateau level very likely represents the total amount of the LMWP that accumulates in the kidney, since the ^{131} I-TC label remains trapped in the tubular cell. The rate of uptake appears to be similar to the uptake kinetics of ^{123}I-LMWP. This indicates that the difference in the two labels does not affect the renal uptake of the LMWP.

Total amount of renal LMWP

The total amount of renal uptake was obtained in two different ways. First of all, by extrapolating the renal degradation curve of ^{123}I-LMWP to the time of injection. This is visualized in figure 2 by the percentage of injected dose at time zero on the regression line of degradation (A). Secondly, by estimating the renal accumulation of ^{131}I-TC-LMWP at the plateau level (figure 2, visualized by the horizontal line to time zero: B). The results are listed in table 3. The data show that the two methods are in good agreement for the proteins, cytochrome-c and aprotinin. For lysozyme, the ^{123}I uptake data are lower ($p = 0.05$) and exhibit more variation (49-84%) compared to the ^{131}I-TC results (77-98%). Striking is the high and remarkably similar renal accumulation of the three LMWPs: 88, 84, 79% of the injected dose for ^{131}I-TC labeled cytochrome-c, lysozyme and aprotinin, respectively.

LMWP	RENAL UPTAKE (%) ^{123}I-LMWP	^{131}I-TC-LMWP
cytochrome-c	89 ± 4 (72-108)	88 ± 5 (67-99)
lysozyme	67 ± 6 (49-84)	84 ± 3 (77-98)
aprotinin	78 ± 2 (69-84)	79 ± 3 (70-90)

Table 3. *Total amount of renal uptake (% of injected dose) of radiolabeled LMWPs after intravenous injection in 6 rat.* Data are given as mean ± SEM (values in parenthesis denote the range). Data are obtained by kinetic analysis of the renal time-course of ^{123}I and by estimating the stable maximum level of the renal ^{131}I radioactivity.

Degradation products

Plasma samples were analyzed for TCA-soluble radioactivity, representing the degradation products of the intravenous injected radiolabeled LMWP. No detectable amounts of ^{131}I degradation products were found in the plasma suggesting that the ^{131}I-TC label is indeed trapped in the cell in which it is internalized. This is in contrast to the ^{123}I degradation products, of which the plasma appearance pattern (panels B, figure 1) seems to be in agreement with the rate of LMWP degradation in the kidney (figure 2).

The urine accumulation of radioactivity was monitored continuously by external counting. Subsequently, urine samples were analyzed for protein, amino-acid bound and unbound radioactivity by TCA precipitation and silver crystallization. Like in plasma, no detectable amounts of ^{131}I-TC-degradation products were found in the urine. Figure 3 shows the accumulation of the ^{123}I degradation products in the urine. Since the kidney is the major site of LMWP degradation, supposedly the ^{123}I degradation products in the urine are mainly originating from the kidney. Indeed, the urine appearance of the ^{123}I degradation products appears to coincide with the renal disappearance of ^{123}I (figure 2). During the time of the study with cytochrome-c, lysozyme and aprotinin, 73 ± 3%, 61 ± 2% and 43 ± 3% of the injected ^{123}I was released from the kidney, respectively. In the same period 44 ± 3% (Cy), 40 ± 4% (Ly) and 25 ± 2% (Ap) of the injected dose of ^{123}I was recovered as degradation products in the urine. This implies that 60% (Cy), 66% (Ly) and 59% (Ap) of the degradation products released from the kidney during the time of the study were recovered in the urine. This maybe due to the large distribution volume of the ^{123}I-breakdown products in the body. From these degradation products 67 ± 5% (Cy), 73 ± 5% (Ly) and 91 ± 1% (Ap) were bound to amino-acids. The rest was free ^{123}I. This indicates that the efficiency of deiodinase inhibition by the MIT was not complete and different for the three LMWPs studied. No detectable amounts

of LMWP bound iodine were found in the urine, suggesting that the injected tracer amounts of LMWPs were maximally reabsorbed by the proximal tubular cell.

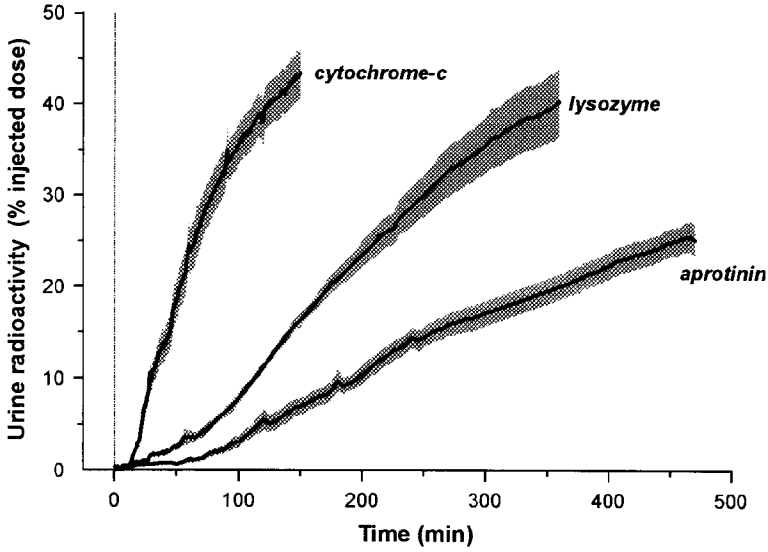

Figure 3. *Time-course of urinary excretion of ^{123}I after intravenous injection of ^{123}I radiolabeled cytochrome-c, lysozyme and aprotinin in 6 rats.* Values are given as mean ± SEM. More than 95% of the ^{123}I appeared to be TCA soluble, representing radiolabeled degradation products. No ^{131}I was recovered in the urine.

Discussion

In the present study, we show that we can quantify both renal uptake and degradation of a ^{123}I-radiolabeled LMWP in an intact rat by registering the renal time-activity curve with a gamma-camera and applying subsequent kinetic analysis to the obtained curve.

The total amount of renal uptake was approximately 80% of the administered dose for all the three LMWPs, cytochrome-c, lysozyme and aprotinin. An accumulation up to 80% of the injected dose is in good agreement with some of the studies in the literature. First of all, our results are in line with the renal extraction studies, which show 73% extraction for parathyroid hormone [2], 67% for growth hormone [3] in rats, and 90% for growth hormone in sheep [4]. However, such studies may show considerable variability since they are based on a combination of *in vivo* and kidney perfusion data [2,3] or on measurements of minute differences in protein concentrations in renal arterial and venous blood [4]. Secondly, our

uptake data match the 90% contribution of the kidney in the catabolism of lambda-l-chain reported by Waldmann et al. [5]. These authors calculated this value using the metabolic rate of the LMWP and the creatinine clearance in healthy individuals.
A more direct way to study the renal handling of LMWPs is the measurement of the actual amount of LMWP in the kidney. In spite of the similar experimental approach, most of such studies suggest a markedly lower renal uptake and differences among the various LMWPs compared to the 80% for all the three LMWPs in the present study. What are the possible explanations for these differences? Just, Christensen, Ottensen and Bianchi et al. [6-9] injected iodinated LMWPs intravenously in the rat and measured the radioactivity in extirpated kidneys after different time-intervals. Depending on both the LMWP and on the time point with the highest radioactivity, 29-58% of the injected dose was maximally recovered in the kidneys. However, this renal radioactivity is likely to be an underestimation of the total amount of LMWP uptake. First of all, it is difficult to determine the exact time point on which the renal amount is maximal. Bianchi solved this by establishing the maximum beforehand by continuous external counting [9]. Secondly, the level of this "maximum" is determined by both uptake and degradation of the LMWP in the kidney. In our study with ^{123}I-LMWP we bypassed these limitations, since our kinetic analysis of uptake is based on correction for the renal rate of degradation of the ^{123}I-LMWP.
To validate the calculated amount of renal uptake of ^{123}I-LMWP, we co-injected ^{131}I-TC-LMWP. Since the ^{131}I-TC label remains trapped in the kidney cell, there will be no interference with the renal degradation of the LMWP. This method was used before by Hysing et al. [10]. Compared to us, they found the rather low renal uptake values of 43% for cytochrome-c and 35% for lysozyme. Although we used the same procedures of iodination and coupling, we did not detect any radioactivity in the liver. This in contrast to their study in which a hepatic accumulation of 19% of the injected dose was found. Such an hepatic uptake may indicate denaturation or aggregation of the protein. Besides this, other factors should be considered like the anesthesia and the computation of the amount of radioactivity in the extirpated kidney relative to the administered dose. In our study, the reliability of the ^{131}I-TC-LMWP results largely depends on the proper corrections of the gamma-camera data. However, evidence that these corrections did not significantly effect our uptake data is shown by the very similar data of the separate study with ^{123}I-TC-lysozyme in which no corrections are needed.
Apart from the total amount of renal uptake, we quantified the rate of renal uptake and degradation of the LMWP. There is a clear difference in the rate of renal uptake between the three LMWPs. Differences in renal extraction appear not to explain this phenomenon, since the renal clearance of the three LMWPs are

reported to be very similar and close to the glomerular filtration rate [1]. In this study, the renal clearance, calculated from the renal rate of uptake and the distribution volume, appeared to be 1.9, 1.0 and 1.1 ml/min for cytochrome-c, lysozyme and aprotinin, respectively. In agreement with the literature, these data are indeed quite comparable to one another and close to the glomerular filtration rate, which makes it rather unlikely that the difference in renal extraction explains the difference in rate of renal uptake. This leaves open the option of a difference in extra-renal distribution volume between the different LMWPs. Evidence for this option is clearly shown by the difference in estimated distribution volume of the three LMWPs.

The renal rate of degradation of the three LMWPs, cytochrome-c, lysozyme and aprotinin have not been quantified before. The estimated data from the literature are in line with our results. The rate of renal degradation of cytochrome-c is high (within minutes) [7,9,11], of lysozyme intermediate [8,9], and of aprotinin low (several hours) [9].

In conclusion, we show that the renal handling of LMWPs can be quantified non-invasively in the intact rat by applying kinetic analysis on the renal time-activity curve of ^{123}I-labeled LMWP, obtained with a gamma-camera.

For the three tested LMWPs, cytochrome-c, lysozyme and aprotinin the total amount of renal uptake is high (about 80%) and strikingly similar, whereas their renal degradation rate is quite different. The presented technique may not only be used to extend our knowledge in renal LMWP handling in animal experimental settings, but may also be of use in a clinical environment.

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Chapter 3

Quantification of renal low-molecular-weight protein degradation in the intact rat

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Introduction

The process of low-molecular-weight protein (LMWP) catabolism in the kidney has been studied extensively. Morphological and biochemical studies have established that the main pathway is endocytosis of filtered LMWPs by the proximal tubular cells followed by an intracellular migration via endosomes to proteolytically active lysosomes. Within the lysosomes the LMWPs are degraded into small peptides and single amino-acids [1-3]. Although this mechanism of renal elimination is similar for the majority of LMWPs, large differences have been found for the individual proteins with regard to degradation rate. Previous studies on this topic were performed either in-vitro kidney preparations [2-6] or by measuring the renal LMWP concentration in extirpated kidneys at different time-intervals [2,7-9]. The disadvantage of the latter in-vivo studies is the low time-resolution. Recently, Bianchi et al used an external counting technique, which monitored the radiolabeled LMWP over the kidneys of the anesthetized rat. This enabled measurement of the renal content in time with a high time-resolution [7]. Unfortunately, the decrease of radioactivity after initial uptake was monitored for only 10 min. In this short period of time, the descending phase reflects ongoing renal uptake and simultaneous degradation of the LMWP. Therefore, a reliable parameter for the actual degradation data could not be obtained.
In this paper we focus on the quantification of the renal degradation of ^{123}iodine radiolabeled LMWPs in the intact rat by external counting. The radioactivity in the kidney predominantly represents the labeled-LMWP because labeled-breakdown products are secreted rapidly from the renal tubular cell into the blood compartment [1]. The high sensitivity of the external counting technique enabled us to quantify the degradation by computer kinetic analysis. The three LMWPs, cytochrome-c, lysozyme, and aprotinin were chosen on the basis of their alleged differences in renal half-life.

Materials and Methods

Materials
Cytochrome-c (horse heart; MW 12,400; pI 10.6), Lysozyme (eggwhite; MW 14,300; pI 11) and Aprotinin (bovine lung; MW 6,500; pI 10.5) were obtained from Sigma (Axel, The Netherlands).

^{123}I-LMWP synthesis

^{123}I-LMWP labeling was performed according to the Chloramine-T method of Hunter [10]. In short, 50 ml protein (10 mg/ml) and 10 ml Chloramine-T (5 mg/ml) were added to 37 MBq sodium-^{123}iodine in 100 ml phosphate buffer (0.2M, pH 7.4) and mixed for 60 seconds. The reaction was stopped by addition of 25 ml metabisulfite (2.5 mg/ml). Free iodine was removed by a separation on a Sephadex G25 column. The protein fraction was used within two hours.

Experimental set-up

A week before the experiment female Wistar rats were instrumented with a permanent venous heart canule. The day before the experiment, the animals were fasted overnight and received 2 mg sodium-iodine intravenously to minimize the accumulation of unbound iodine in various organs. Anesthesia was induced with halothane and a single dose of 30 mg/kg pentobarbital. During the experiment anesthesia was maintained by continuos infusion of 4 mg/kg/h pentobarbital. Furthermore, the rats received a continuous infusion of 2 ml/h 5 % glucose and 6 mg/h 3-monoiodo-l-tyrosine (MIT) to achieve a stable urine production and a saturation of the deiodases [11,12], respectively. Three rats were fixed on a middle-energy collimator of a gamma-camera. A window was selected on the peak energy of the ^{123}I with a width of 150 keV. Body temperature was monitored and controlled with a heat-pad and a lamp. Urine was collected continuously with a short bladder canula ending in a tube situated on the collimator. After a stabilization of one hour, 1.1 MBq ^{123}I-LMWP was injected intravenously. The gamma-camera recorded the activity in one or five minute intervals for 3 to 8 hours, depending on the protein studied. The radioactivity time course of the right kidney, urine and total body was subsequently plotted after analysis of the respective "regions of interest".

Pharmacokinetic and statistic analysis

The pharmacokinetic analysis was performed using a computer program for non-linear curve-fitting, MultiFit (University of Groningen, Department of Pharmacology and Therapeutics, Groningen), using the simplex algorithm [13]. Initial parameter estimates are automatically obtained by a curve-stripping procedure. The reciprocal of the y-value predicted by the model was used as the weighing factor [14].

All data are expressed as mean $\pm$ SEM. Statistical analysis was performed with the Wilcoxon rank test.

Results

Renal time course

The renal time-courses of radioactivity shown in the figure depicts the renal handling of the three ^{123}I labeled LMWPs. After the first pass uptake, the renal uptake continued gradually in combination with a degradation of the LMWP in the kidney. To verify that ^{123}I breakdown products were not retained within the kidney, thus obscuring our degradation data, we measured the amount of lysozyme-bound ^{123}I in extirpated kidneys at the end of the experiment: 84 ± 2 % (n=3) of the ^{123}I present in the kidney appeared to be bound to lysozyme.

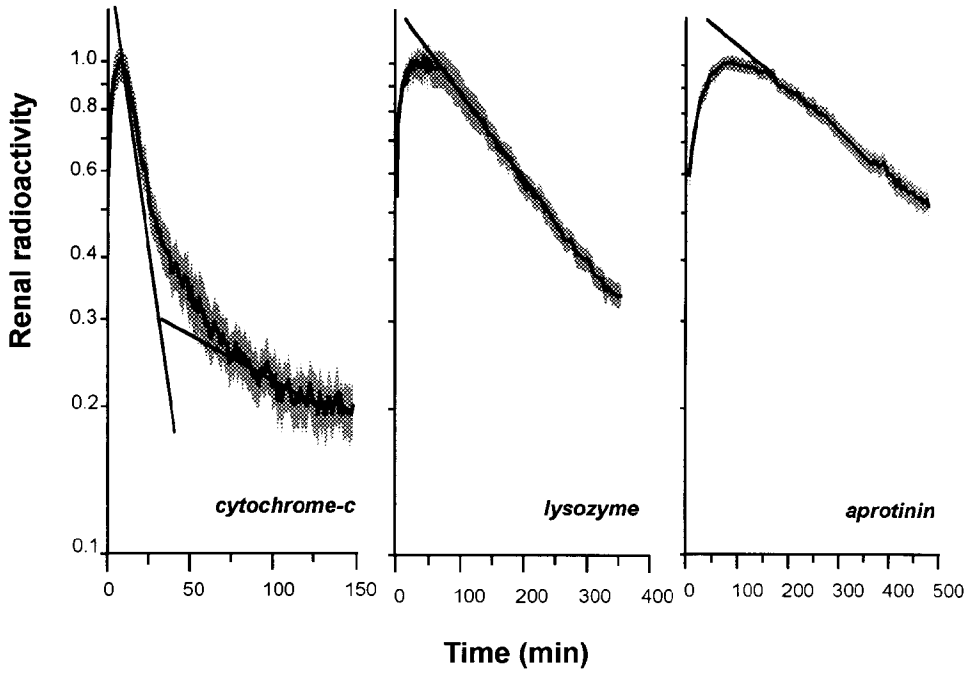

Figure. *Renal time-course of radioactivity with the kinetically fitted line(s) of renal elimination of radioactivity.* Cytochrome-c curve is the average of 5 rats. Lysozyme and aprotinin curves are the average of 6 rats.

The kinetic analysis of the renal time-course of the three LMWPs, shown in the figure reveals a marked difference in breakdown between the three LMWPs. The degradation of lysozyme and aprotinin was linear throughout the time of the study

while the degradation of cytochrome-c was biphasic. The renal half-life of lysozyme and aprotinin was 252 ± 26 and 443± 28 min, respectively. Cytochrome-c was degraded with a half-life of 10.6 ± 1.3 and of 222 ± 54 min. The half-life of lysozyme, aprotinin and the first of cytochrome-c were significantly different from each other ($p < 0.01$).

Urine

The urine, collected in the tube placed on the gamma-camera, showed increasing amounts of radioactivity in time. The urine accumulation curve paralleled the renal degradation curve for each LMWP. Upon analysis of the urine, no detectable amounts of TCA-precipitable radioactivity was found in the urine. This indicates that the tracer amount of LMWP was completely reabsorbed by the renal tubules. The urine radioactivity appeared to consists of free ^{123}I and ^{123}I-tyrosine, the known breakdown products of the ^{123}I-LMWPs.

Discussion

The present study shows that the renal degradation of radiolabeled low-molecular-weight proteins can be elegantly monitored in-vivo using a gamma-camera. Because of the high time resolution of the obtained data, a kinetic analysis could be performed giving an accurate estimate of both the renal half-life as well as potential rate limiting factors in the degradation process. A perquisite for applying this method is that the externally registered renal radioactivity only reflects the actual amount of more or less intact LMWP in the tubule. Thus, breakdown products should not be retained in the kidney. We showed that the vast majority of the radioactivity in the kidney was protein-bound. According to Maack et al.[1] this is independent on the time after LMWP accumulation. Another confounding factor could be that the apparent fall in renal radioactivity is determined by the de-iodination of the intact LMWP. This however seems unlikely. First of all, Christensen and Bertolatus et al. have shown that deiodinases only act on small peptide structures and amino-acids [11,12]. Secondly, we infused relatively large amounts of mono-iodo-tyrosine to saturate the deiodinases.

Our data show that the degradation kinetics of the three LMWPs are markedly different. How can these differences be explained? The molecular weight and degradation rate of the LMWPs seem unrelated, since the renal half-life of cytochrome-c (10 min), lysozyme (252 min), and aprotinin (443 min) do not correlate with the respective molecular weights (12,400; 14,300 and 6,500 Dalton, respectively). An effect of the overall charge, in the acid environment of the

lysosomes is unlikely because all three LMWPs are cationic (pI: ± 11). Our data confirm those of Bianchi et al. who studied the renal time-course of 8 different LMWPs by external counting and found no relation between estimated breakdown and molecular weight or pI [15]. However, other substance specific factors could have obscured such a relation. For example, the endosomal migration time from the tubular lumen to the lysosomes in the proximal tubules cells could bias the actual degradation rate of an LMWP. Indeed, Christensen and Hysing et al. showed that cytochrome-c is accumulated in the lysosomes within 3 minutes, whereas lysozyme seemed to migrate for 20 minutes before the degradation started [2,8]. The long half-life of aprotinin, an inhibitor of proteolytic enzymes, could possibly be explained by an inhibition of its own degradation, a suggestion made by Bianchi [7]. Another interesting observation is the biphasic nature of the cytochrome-c breakdown, suggesting the presence of at least two different tubular degradation pathways. Several explanations for this biphasic degradation can be given. First of all, in-vitro studies have shown that insulin is degraded both inside and outside the lysosomes with probably different degradation rates [16]. This could also be the case for cytochrome-c. Secondly, the radiolabels, we used, may well be attached to different parts of the molecule, which in turn may result in a difference in formation of the I-breakdown products. Again for insulin, it has been demonstrated that degradation may indeed start at one particular molecular site followed by degradation at the remaining site [16]. Apart from these physiological explanations, we have to consider certain artifacts that could have caused the biphasic nature of the cytochrome-c degradation. Although we used a mild iodination procedure, we can not rule out that a part of the ^{123}I-radiolabeled cytochrome-c batch was denatured, and that this material exhibited an increased or decreased degradation rate. However, in that case we would have expected some hepatic accumulation, which was not observed. Furthermore, the degradation of cytochrome-c appeared to be very fast which resulted in a temporary accumulation of breakdown products in the circulation (TCA-soluble radioactivity was found in the blood). Therefore, we also examined whether the second phase of the renal radioactivity decay curve could be the result of urinary excretion of circulating breakdown products instead of degradation of the cytochrome-c in the kidneys. To check the influence of this process, we repeated the kinetic analysis with renal radioactivity data that were corrected for an estimated background. However, this process did not have a major impact on our kinetic analysis, since estimated background correction of the data did not reveal significantly different half-life ($t_{1/2}$:9.2 and 204 min). Thus, the biphasic descending phase may indicate a second (minor) pathway of cytochrome-c degradation.

In summary, this study shows that the in-vivo renal handling of iodinated LMWPs can be qualified and quantified using an external counting method in the rat. The thus observed large differences in renal kinetics of the lysozyme, aprotinin and cytochrome-c appear not to be related to their molecular size or charge, and remain as yet unexplained.

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Chapter 4

Proteinuria associated changes in the renal handling of low-molecular-weight proteins in the rat

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Abstract

A direct consequence of glomerular protein leakage is an increased tubular exposure to proteins. The aim of the present study was to examine whether the proximal tubular function is progressively affected by increasing levels of chronic proteinuria. For this purpose, the renal uptake and catabolism of segment-specific low-molecular-weight proteins (LMWPs) (cytochrome-c for the S_1 segment and myoglobulin for the S_2 segment) were determined in rats with different degrees of proteinuria. Male Wistar rats were exposed to a single dose of adriamycin intravenously (2 mg/kg) to induce proteinuria. Proteinuria ranged from 100 to 1200 mg/day after 3 weeks. The body distribution of the intravenously injected radiolabeled LMWPs was recorded by external counting under anesthesia. The effect of proteinuria on renal uptake of the two LMWPs was small with an uptake reduction of 1.5 $\pm$ 0.5 % and 1.3 $\pm$ 0.3 % of the injected dose for cytochrome-c and myoglobulin respectively with every 100 mg/day of proteinuria increase ($p < 0.01$). The effect of proteinuria on renal rate of catabolism of the two LMWPs was more pronounced and highly correlated ($p < 0.0001$) with a reduction in cytochrome-c catabolism of 1.7 $\pm$ 0.3 %/30 min and in myoglobulin catabolism of 3.0 $\pm$ 0.5 %/30 min with every 100 mg/day increase in proteinuria. The rate of myoglobulin catabolism was significantly more affected by proteinuria than that of cytochrome-c ($p < 0.01$). These data suggest that proteinuria-induced damage of the proximal tubulus is specific for particular tubular functions and tubular segments. The function of protein catabolism is more affected than the function of protein uptake. In addition, the S_2 segment is more affected than the S_1 segment. These data may provide new insight into the detrimental effects of proteinuria within the nephron.

Introduction

Proteins that are filtered in the glomerulus are reabsorbed by the proximal tubular cell and subsequently catabolized in the intracellular lysosomes [1,2]. In normal physiological circumstances, mainly low-molecular-weight proteins (LMWPs) are handled by this system. During glomerular disease however, larger proteins such as albumin leak through the glomerular filter, leading to an increased burden for the proximal tubular system. This tubular protein overload affects the physiological state of the tubular cell, and could well be the pathophysiological link between glomerular protein leakage and tubulointerstitial damage leading to progressive renal function loss [3].
The tubular handling of a LMWP may be used as indicator of the physiological state of the proximal tubule. Clinical and experimental studies have shown that during severe glomerular damage the total renal uptake [4,5] and total renal catabolism of LMWPs is reduced [5], whereas proteinuria was associated with an increased catabolism of a LMWP [6]. Ex-vivo studies showed that tubular lysosomal enzyme activity is increased during mild proteinuria suggesting an increased protein catabolism [7-9]. In contrast, the tubular lysosomal enzyme activity is reduced during chronic high proteinuria suggesting that severe chronic proteinuria causes a reduction in protein catabolism [7,10]. Apparently, the effect of proteinuria on tubular function is complex and depends on the severity and duration of the protein leakage. One study showed a correlation between severity of experimental proteinuria and the degree of tubular protein uptake as measured by urinary excretion of LMWPs [4]. No data are available on the relationship between the severity of proteinuria and the renal uptake of LMWPs during chronic proteinuria, whereas a possible correlation between renal protein catabolism and proteinuria is known.
Recent studies have shown an axial heterogeneity in protein handling along the proximal tubule. The LMWP lysozyme and the sex-dependent LMWPs named α_{2u}-globulins are predominantly taken up by the early proximal convoluted tubules (S_1 segments) [11,12] whereas myoglobulin appears to be taken up primarily in the late proximal convoluted tubules (S_2 segments) [12]. In the nonproteinuric state, tubular infused albumin is evenly reabsorbed along the proximal tubules [11]. No data are available about the axial heterogeneity during proteinuria except that lysosomal enzyme activity studies suggest that a temporary and low degree proteinuria causes an additional albumin handling in the S_2 segment compared to the nonproteinuric state [7,8].
The aim of the present study was to examine whether the function of the proximal tubule, measured as LMWP uptake and catabolism, is affected by

chronic proteinuria and whether the degree of proteinuria determines the degree of functional alterations. Secondly, we studied the effect of proteinuria on these tubular functions for the different segments of the proximal tubule. Adriamycin nephrosis in the rat was chosen as a model of stable proteinuria with a wide range of protein excretion. To determine the segmental differences, the renal handling of the S_1 specific LMWP cytochrome-c was compared with that of the S_2 specific LMWP myoglobulin [12,13].

Material and Methods

Materials

Cytochrome-c (horse heart; molecular weight 12,400; pI 10.6) and myoglobulin (horse heart; molecular weight 17,800; pI 7,3) were obtained from Sigma (Axel, The Netherlands). Adriamycin.HCL (2 mg/ml injection solution) was purchased from Farmitalia Carlo Erba (Brussels, Belgium).

Experimental set-up

Male Wistar rats (Harlan, Zeist, The Netherlands) weighing about 250 g were kept on a low sodium diet (0.05 % NaCl/20 % protein, Hope Farms Inc. Woerden, The Netherlands). Rats were anesthetized with isoflurane 2 %/O_2 (500 ml/min) and injected with 1.5 mg/kg adriamycin via the dorsal penal vein to induce proteinuria. Five rats did not receive adriamycin and were used as nonproteinuric controls. Once a week, 24 hour urine samples were collected to measure the course of proteinuria. Three weeks after induction of proteinuria, the adriamycin injected rats were divided in two groups stratified for similar proteinuria range. One group was used to study the renal handling of cytochrome-c (n = 9) and one group to study the renal handling of myoglobulin (n = 9). The renal handling of the two LMWPs was measured at week 3 and 5 after induction of proteinuria, this to measure the effects in the individual rat during 2 different levels of proteinuria [14]. Unfortunately, this aim was not reached since the increase in individual proteinuria going from week 3 to 5 was only 22 ± 12 % for the cytochrome-c group and 24 ± 6 % for the myoglobulin group. Of the nonproteinuric controls, 2 rats were used to study the renal handling of cytochrome-c and 3 to study myoglobulin. Gamma-camera studies were carried out according to a previously described protocol [15]. In short, during anesthesia isoflurane 2 %/O_2 500 ml/min) the rats were fixed on a low-energy collimator. A window was selected on the energy peak of ^{123}iodine with a width of 250 keV. Body temperature was monitored and maintained on 37°C

with a heat pad and a lamp. After a stabilization of 10 min, approximately 2 MBq ^{123}I-LMWP was injected within 15 seconds via the dorsal penal vein. The gamma camera recorded the radioactivity in 1 minute frames for 1 hour. The radioactivity time course of the right kidney and total body was plotted after analysis of the respective "regions of interest". Data on the left kidney were not analyzed because of cumulated radioactivity in the stomach region possibly due to accumulation of free iodine [16]. Renal uptake of tracer was related to total body radioactivity as 100% of the injected dose, corrected for counting efficiency [15].

Radiolabeling

^{123}I-LMWP labeling was performed according to the chloramine-T method of Hunter and Greenwood [17]. In short, 50 µl protein (10 mg/ml) and 10 µl chloramine-T (5 mg/ml) were added to 20 MBq (cytochrome-c) or 40 MBq (myoglobulin) Na^{123}I in 100 µl phosphate buffer (0.2 M, pH 7.4) and mixed for 60 seconds. The reaction was stopped by addition of 25 µl metabisulfite (2.5 mg/ml). Free iodine was removed by separation on a Sephadex G25 column. The labeling efficiency was 98 % for cytochrome-c and 42 % for myoglobulin. The protein fraction was kept on ice until use. At the start and end of the study respectively, 95 %, 92 % (cytochrome-c) and 81 %, 75 % (myoglobulin) of the iodine was protein bound. All rats were randomly studied during a single day.

Data analyses

Since in the present study major individual differences in proteinuria between week 3 and 5 were not achieved, the individual data of week 3 and 5 were handled as independent values for interindividual comparison (each rat was thus included twice). The relationship between the degree of proteinuria and renal uptake or renal catabolism was analyzed by linear regression and the value at proteinuria of 0 mg/day was determined by extrapolation to the y-intercept. The rats were divided in a low proteinuria (< 600 mg/day) and high proteinuria (> 600 mg/day) group and the individual data of renal uptake and catabolism expressed as percentage of the value in nonproteinuric state.

All comparisons were made using the Student's t-test. Data are presented as mean ± SEM except for the data obtained from linear regression analysis which are expressed as mean ± SE.

Result

Rats were successfully stratified for a similar proteinuria range 3 weeks after proteinuria induction. Proteinuria in the cytochrome-c group ranged between 170 and 1050 mg/day with a mean of 529 ± 121 mg/day and in the myoglobulin group between 105 and 1010 mg/day with a mean of 538 ± 116 mg/day.

Renal uptake

Figure 1 shows the individual time courses of renal radioactivity after administration of ^{123}I-cytochrome-c and ^{123}I-myoglobulin at week 3 and 5 after induction of proteinuria. Only the first 10 min of the curves are presented in order to highlight the individual differences in renal uptake of the proteins. The maximal accumulation of radioactivity was used to express the protein uptake capacity of the kidney. The renal uptake ranged between 41 % and 74 % of the injected dose for cytochrome-c and between 47 % and 70 % of the injected dose for myoglobulin.

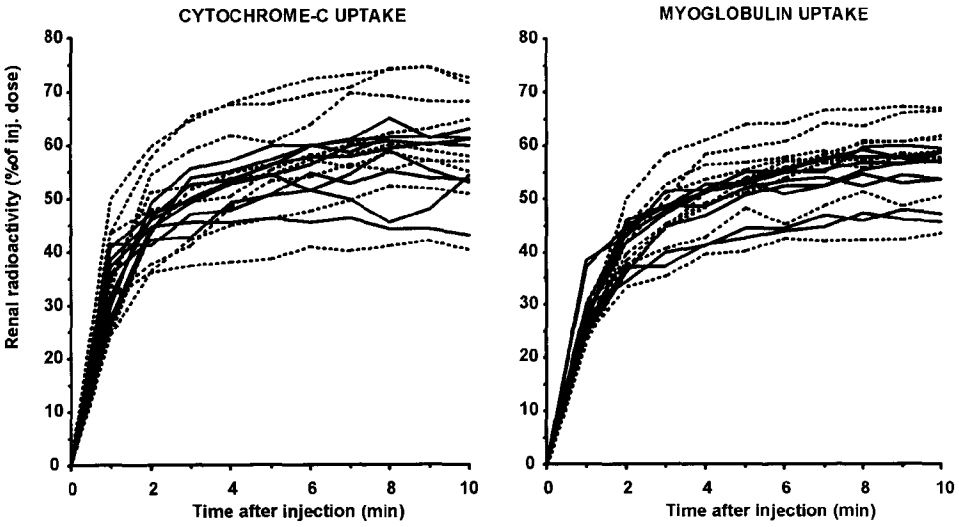

Figure 1. *Individual time courses of renal ^{123}I-cytochrome-c and ^{123}I-myoglobulin, expressed as percentage of injected dose.* Dotted lines represent the time-courses recorded 3 weeks after induction of proteinuria (n = 9), the solid lines 5 weeks after proteinuria induction (n = 9).

Figure 2 shows the relationship between proteinuria and the renal uptake of cytochrome-c and myoglobulin. Increasing proteinuria correlated significantly with a lower renal uptake of the two low-molecular-weight proteins (LMWPs)

($p < 0.01$). Renal uptake of cytochrome-c reduced with 1.5 ± 0.5 % of the injected dose and of myoglobulin with 1.3 ± 0.3 % of the injected dose per 100 mg/day increase in proteinuria. Extrapolation of the correlation lines to the y-intercept indicated a renal uptake of 68 ± 3 % of the injected dose cytochrome-c and 65 ± 2 % of the injected dose myoglobulin at zero proteinuria. These extrapolated values to the nonproteinuric state are in good agreement with those found in rats not injected with adriamycin. In two normal, nonproteinuric rats, renal uptake of cytochrome-c was 79 % and 63 % of injected dose which is in agreement with a previous study [15]. Renal uptake of myoglobulin in three nonproteinuric rats was 67 ± 2 %.

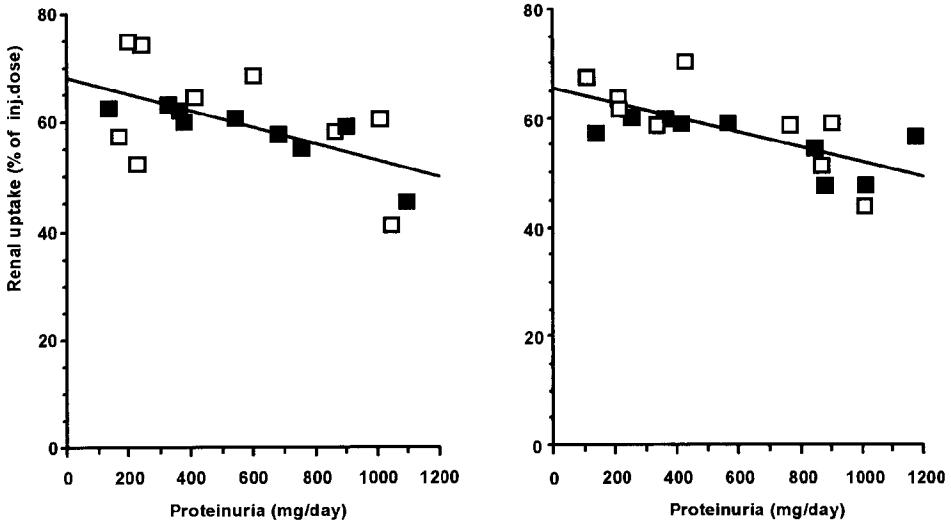

Figure 2. *Relationship between proteinuria and renal uptake of cytochrome-c and myoglobulin.* Renal uptake is expressed as maximal amount of renal radioactivity in percentage of injected dose. Rats were studied 3 weeks (open squares) and 5 weeks (closed squares) after induction of proteinuria.

Renal degradation

The individual renal rates of catabolism of myoglobulin and cytochrome-c at week 3 and 5 are visualized in figure 3. The curves show that renal radioactivity decreases in time after reaching a maximum. This indicates that the radiolabeled proteins are catabolized in the kidney with a subsequent renal release of the formed radioactive breakdown products [18]. The difference in renal radioactivity between the time of maximal renal accumulation and 30 min later

was used to express the rate of LMWP catabolism in the kidney. The individual rates of catabolism were highly variable. Renal catabolism ranged between 26 %/30 min and 53 %/30 min for cytochrome-c and between 13 %/30 min and 56 %/30 min for myoglobulin.

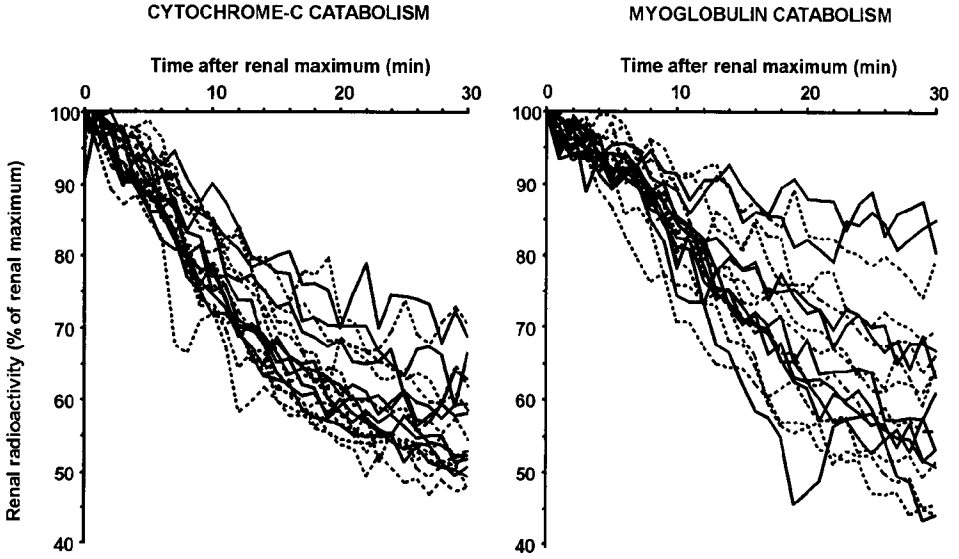

Figure 3. *Individual time courses of renal ^{123}I-cytochrome-c and ^{123}I-myoglobulin, expressed as percentage of maximal renal amount.* Dotted lines represent the time-courses recorded 3 weeks after induction of proteinuria (n = 9), the solid lines 5 weeks after proteinuria induction (n = 9).

Figure 4 shows the relationship between proteinuria and the renal catabolism of cytochrome-c and myoglobulin. Increasing proteinuria correlated significantly with a lower renal catabolism of the two LMWPs ($p < 0.0001$). Renal catabolism of cytochrome-c reduced with 1.7 ± 0.3 %/30 min and of myoglobulin with 3.0 ± 0.5 %/30 min with every 100 mg/day increase in proteinuria. Extrapolation of the correlation lines to the y-intercept indicated a cytochrome-c catabolism of 51 ± 2 %/30 min and a myoglobulin catabolism of 55 ± 3 %/30 min in nonproteinuric state. These calculated values of the nonproteinuric state are in good agreement with, or at least not lower than, the determined values of rats not injected with adriamycin. The renal catabolism of cytochrome-c in two nonproteinuric rats was 46 %/30 min and 51

%/30 min which is in agreement with a previous study [15]. The range of renal catabolism of myoglobulin in three nonproteinuric rats was 47 ± 6 %/30 min.

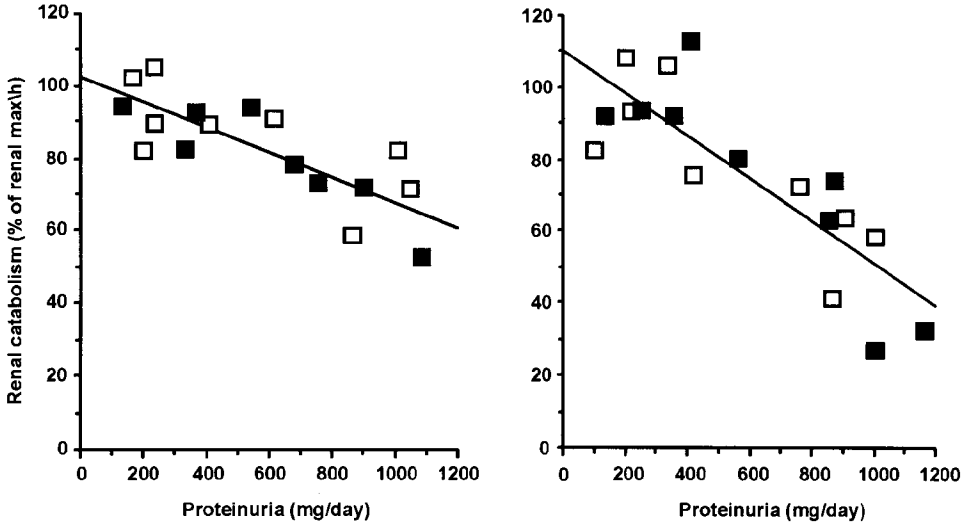

Figure 4. *Relationship between proteinuria and renal catabolism of cytochrome-c and myoglobulin.* Rate of renal catabolism is expressed as percentage of decrease in renal radioactivity between the time of maximal amount of renal radioactivity and 30 min later. Rats were studied 3 weeks (open squares) and 5 weeks (closed squares) after induction of proteinuria.

Average data of low and high proteinuria groups

For statistical comparison, the adriamycin injected rats were divided in a low proteinuria (< 600 mg/24 h, n = 10) and high proteinuria (> 600 mg/24 h, n = 8) group. Mean proteinuria in the cytochrome-c low proteinuria group was 298 ± 40 mg/day and in the myoglobulin low proteinuria group 303 ± 45 mg/day. In the cytochrome-c high proteinuria group, proteinuria was 869 ± 63 mg/day and in the myoglobulin high proteinuria group 933 ± 45 mg/day. The renal uptake of myoglobulin and renal catabolism of myoglobulin and cytochrome-c were significantly affected by severe proteinuria (figure 5). Compared to the low proteinuria group, renal uptake of cytochrome-c was 12 % (not significant) and of myoglobulin 15 % lower ($p < 0.001$) in the high proteinuria group. Renal catabolism of cytochrome-c was 22 % lower ($p < 0.001$) and of myoglobulin 42 % lower ($p < 0.0001$) in the high proteinuria group compared to the low

proteinuria group. In the high proteinuria group, renal catabolism of cytochrome-c was 1.2 times (not significant) and of myoglobulin 1.7 times ($p < 0.001$) more affected by proteinuria than the renal uptake of the two LMWPs. The rate of myoglobulin catabolism was 1.5 times ($p < 0.01$) more affected than of cytochrome-c in the high proteinuria group.

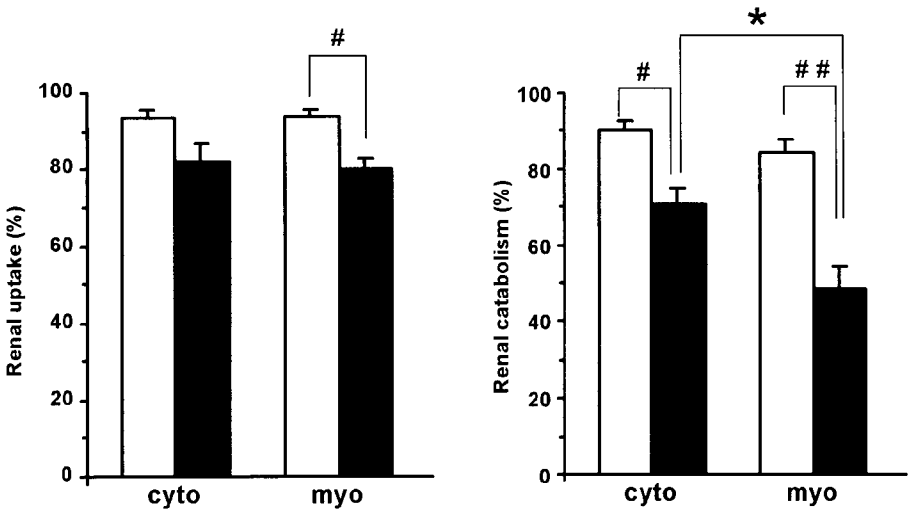

Figure 5. *Renal uptake and catabolism of cytochrome-c and myoglobulin in high and low proteinuria rats.* Rats were divided in a low proteinuria group (< 600 mg/24 h) and a high proteinuria group (> 600 mg/24 h). The data are expressed as percentage of the calculated nonproteinuria value obtained from the correlation between renal uptake or catabolism and proteinuria. White bars represent the low proteinuria group (week 3 + 5: n = 10). The black bars represent the high proteinuria group (week 3 + 5: n = 8). Data are expressed as mean + SEM, * $p < 0.01$, # $p < 0.001$, # # $p < 0.0001$.

Discussion

The present study shows a relatively small effect of adriamycin induced proteinuria on the renal uptake of the low-molecular-weight proteins (LMWPs) cytochrome-c and myoglobulin. This indicates that damage of the tubular uptake system or competition with the massive amount of filtered protein during proteinuria is negligible in the process of tubular S_1 and S_2 selective LMWP uptake. In contrast, the rate of renal catabolism of the LMWPs was markedly affected suggesting that proteinuria interferes in the intracellular catabolic

function of the proximal tubular cell. The catabolism of the S_2 specific LMWP myoglobulin was more affected than of the S_1 specific LMWP cytochrome-c indicating a higher susceptibility of the S_2 segment for damage by proteinuria.
How do our renal uptake data compare to current data in the literature? In the present study, the effect of adriamycin induced proteinuria on renal uptake of the LMWPs cytochrome-c and myoglobulin was minimal. Thus, competition for protein uptake or damage of the tubular uptake system by adriamycin or proteinuria appears not to occur. This seems at variance with the study of Thielemans et al. in which adriamycin-induced proteinuria was associated with a more than 100-fold increase in the urinary excretion of endogenous LMWPs [4]. They gave however a higher dose of adriamycin with consequently a more rapid development of massive proteinuria. Maximum albuminuria was already reached in 8 days whereas in our study proteinuria developed gradually and only reached its maximum after 3 weeks. We hypothesize that our results are explained by a tubular cell adaptation to chronic proteinuria by introduction or upregulation of a separate uptake system for the overload of albumin. This suggestion is based on the fact that a separate, fluid phase albumin uptake system has been found in isolated proximal tubules of the rabbit during protein overload [19]. At first glance, this seems in contradiction with the clinical studies of Rustom et al. which showed that in severe glomerular damage and proteinuria, tubular uptake of a LMWP was reduced [5,6]. However, the reduced renal uptake in those studies could not be explained by saturation or damage of the uptake system of the proximal tubular cells since no intact LMWP was recovered in the urine.
The catabolism of the LMWPs myoglobulin and cytochrome-c was progressively reduced with increasing severity of the adriamycin induced proteinuria. This is the first report showing a correlation between the degree of proteinuria and rate of LMWP catabolism in the rat kidney. In humans, both an increase and decrease of total renal LMWP catabolism have been reported, depending on the severity of glomerular disease [5,6]. We consider it unlikely that the reduced LMWP catabolism in our study is caused by direct tubular toxicity of adriamycin since the calculated nonproteinuria value of catabolism obtained from the correlation between renal catabolism and proteinuria was comparable, or at least not lower than the catabolism measured in the rats not injected with adriamycin. Furthermore, Eisenberger et al. argued that adriamycin did not affect lysosomal enzyme activity in isolated proximal tubulus cells [8]. Thus, reduction in catabolism is more likely to be related to proteinuria as such.
Which factor could be responsible for the proteinuria associated reduction in renal catabolism of LMWPs? Several intracellular processes contribute to the

renal catabolism of proteins and may be affected by the protein load. First, the intracellular endosomal transport system of the proteins may be saturated as suggested by histological studies showing protein droplets in the cytosol of proximal tubular cells of adriamycin nephrotic rats [20]. Second, the capacity of the proteolytic enzymes in the lysosomes may be exceeded [1] followed by hypermetabolism of the cell [7-9], presumably at that time the actual cell damaging process starts. As a consequence of a period of hypermetabolism, lysosomal enzyme activity may be reduced due to exhaustion, and the proximal tubular cell eventually may be destroyed [5,7,10]. Proteinuria lowering therapies may be employed to elucidate at which level of proteinuria the reduced LMWP catabolism becomes a result of irreversible cell damage instead of reversible damage and saturation of the tubular system of protein catabolism. Why is the renal catabolism of LMWPs more affected by proteinuria than the renal uptake? As discussed above, several intracellular processes can in principle be involved in reducing the renal catabolism of LMWPs during proteinuria. However, some of them may at the same time reduce tubular uptake. Saturation of the endosomal transport may affect LMWP uptake indirectly as a consequence of a decreased membrane recycling between the endosomes and luminal membrane [2]. Changes in lysosomal enzyme activity and intracellular damage may not necessarily affect the tubular LMWP uptake but in case tubular cells are actually destroyed, LMWP uptake will be affected. Since uptake of the model LMWPs was minimally affected compared to catabolism, we argue that proteinuria did not substantially affect the endosomal trafficking of LMWPs. This indicates that the bulk of albumin is probably transported via an other system than LMWPs. Of note, the degradation of LMWPs was clearly affected which indicates that the LMWPs and albumin are handled in the same intracellular lysosomes. Furthermore, the data suggest that severe tubular cell destruction did not take place in the present study. However, the data may also be explained by a loss of functioning cells in combination with a stimulated LMWP uptake in the remaining functioning tubular cells [5].

The renal catabolism of the S_2 specific LMWP myoglobulin was more reduced by proteinuria than of the S_1 specific LMWP cytochrome-c. This suggests that the increased amount of filtered proteins during proteinuria is predominantly taken up by the S_2 segment of the proximal tubule with an intracellular damaging effect in those cells or that the S_2 segment is particularly vulnerable to the effects of reabsorbed protein. This is in line with the observation that during low, temporary proteinuria the lysosomal enzyme activity of specifically the S_2 segment is increased [7,8] and chronic severe proteinuria is associated

with reduced tubular lysosomal enzyme activity and reduced renal LMWP uptake [4,7,10].

Does our finding have clinical implications? First, the observation of a heterogeneous damaging effect of proteinuria within the tubular cell and along the proximal tubule may be valuable in understanding pathophysiology of progression of proteinuria associated renal function loss. In turn, this may result in different therapeutic intervention strategies. Second, this method of using two different LMWPs to discriminate between the effects on different tubular segments may become a tool in the clinical diagnosis of tubular function loss in relation to progression of renal function loss.

In conclusion, we show that the proteinuria in adriamycin nephrosis is associated with a change in the proximal tubular system of LMWP handling. These data suggest that proteinuria-induced damage of the proximal tubulus is specific for particular tubular functions and tubular segments. The function of protein catabolism is more affected than the function of protein uptake. In addition, the S_2 segment is more affected than the S_1 segment. These data appear to give new insights into the detrimental effects of proteinuria within the nephron and may provide a lead for new intervention strategies.

Acknowledgment

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Chapter 5

Drug-targeting to the kidney: renal delivery and degradation of a naproxen-lysozyme conjugate in-vivo

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Abstract

A renal specific controlled release of an active drug may enable a reduction of the required dose and may provide a reduction of extra-renal toxicity. To achieve renal specific targeting of the NSAID naproxen, the low-molecular-weight protein (LMWP) lysozyme was employed as carrier since it is mainly taken up and catabolized in the proximal tubules of the kidney. A conjugate was synthesized with an average coupling degree of 2 mol naproxen per 1 mol lysozyme in which the drug was directly coupled to the protein via a peptide-bond. First, we investigated whether naproxen conjugation affects the renal disposition of lysozyme. As native lysozyme, the conjugate was predominantly and rapidly (within 20 min) taken up by the kidney. The subsequent decrease in renal content reflecting the renal degradation of the conjugated lysozyme molecules appeared also to be similar to that of native lysozyme with a half life of 4 hours. Second, the effect of lysozyme conjugation on the body distribution of naproxen was studied. An important observation with regard to the aimed reduction in extra-renal side effects, was that no detectable amounts of free naproxen were present in the plasma after administration of conjugate. Conjugation of naproxen to lysozyme resulted in a pronounced (70-fold) increase of naproxen accumulation in the kidney. In agreement with the protein disposition study, the conjugate was rapidly taken up by the kidney and subsequently degraded. In conclusion, renal selective targeting of the NSAID naproxen can be obtained by conjugation with the LMWP lysozyme. This concept of drug delivery to the kidney has the potentials to improve drug efficacy and safety.

Introduction

Drug targeting to the kidney may be of interest for drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) that are beneficial in the kidney [1] but are toxic elsewhere in the body [2]. To achieve renal specific targeting, low-molecular-weight proteins (LMWP) may serve as carriers. They are rather freely filtered and after reabsorption by the proximal tubular cells, catabolized in the lysosomes [3-5]. Our concept is based on the idea that drugs attached to such a protein will also be carried via this intrarenal route into the proteolytic active lysosomes and released from their protein carrier during catabolism.
Franssen et al. [6] tested the feasibility of using the LMWP lysozyme as carrier for the renal delivery of the NSAID naproxen. The study showed that after an intravenous injection of the conjugate in the rat, the plasma disappearance of naproxen-lysozyme conjugate was similar to that of native lysozyme and clearly different from that of unbound naproxen. This indicates that the conjugate is distributed in the body like native lysozyme, thus that the conjugate is predominantly taken up by the kidney. Although the data indicated renal selective targeting, direct evidence for this was not obtained.
In the present study, we measured the renal specificity of a naproxen-lysozyme conjugate directly. Protein (lysozyme) kinetics and drug (naproxen) kinetics were evaluated with special emphasis on the uptake and degradation in the actual target organ, the kidney. The first observation of renal activity of the naproxen-lysozyme conjugate is also presented.

Materials and Methods

Chemicals
Lysozyme, the sodium salt of naproxen (Nap), ketoprofen and N-hydroxysuccinimide (NHS) were purchased from Sigma (Axel, The Netherlands). Dicyclohexylcarbodiimide was obtained from Aldrich Chemica Co. (Gillingham, Dorset, UK). Water was of millipore quality. All other chemicals were of analytical grade. The prostaglandin E_2 enzymeimmunoassay system was obtained from Amersham Life Science ('s-Hertogenbosch, The Netherlands).

Synthesis of naproxen-N-hydroxysuccinimide ester
To obtain a sufficient conjugation with lysozyme, naproxen was preactivated with N-hydroxysuccinimide. The synthesis of the N-hydroxysuccinimide ester

of naproxen (Nap-NHS) was performed according to Anderson, Zimmerman and Callahan [7]. In 70 ml tetrahydrofuran, 2 g (8.6 mmol) Nap and 1.5 g (13 mmol) NHS were dissolved. After cooling down to -15 °C, 2.5 g (12.5 mmol) dicyclohexylcarbodiimide in tetrahydrofuran was added. The mixture was stirred for 1 hr at -15 °C followed by 20 hr at room temperature. The precipitate dicyclohexyl urea was filtered off and the filtrate evaporated in vacuo. The residue was dissolved in ethylacetate and subsequently washed with water, saturated $NaHCO_3$, water and saturated NaCl solution. After drying on Na_2SO_4, the solvent was evaporated. The material was purified by crystallization overnight at 4 °C with EtAc/petroleum ether 40/60 twice. The overall yield was 2.45 g Nap-NHS (87 %) and the molecular weight of the product was verified with mass spectrometry. The melting point was 116 °C.

Synthesis and characterization of the naproxen-lysozyme conjugate

Conjugation between naproxen was based on a peptide-binding between the carboxylic group of naproxen and available free amino-groups of lysozyme. Synthesis and characterization of the naproxen-lysozyme conjugate was basically performed as described by Franssen at al [8]. Briefly, 10.5 mg (30 µmol) naproxen-NHS in 6 ml dioxane was added in a drop-wise fashion to 140 mg (10 µmol) lysozyme in 24 ml (50 mM) Na_2HPO_4,. Subsequently, the solution was stirred for 2 hours. After centrifugation (2000 r.p.m, 10 min) to remove insoluble products, uncoupled naproxen and other small remaining products were removed by separation on the basis of molecular weight differences using a Sephadex G-25 column (Pharmacia, Woerden, The Netherlands) with dioxane/water 80/20 vol/vol eluens. The protein fraction of the column was washed with water and concentrated in an Amicon stirred cell concentrator using an YM3 membrane (Amicon, Beverly, MA, USA). After lyophilization, the conjugate was stored at - 20 °C.

Three methods were used to characterize the conjugate. (1) The average coupling degree of naproxen to lysozyme was determined by measuring the protein concentration [9] in combination with either a direct fluorescence detection of the conjugated naproxen (Spectrofluorometer, model SPF-500 C™, SLM & Aminco) or naproxen analysis after hydrolysis of the conjugate as described in the HPLC section. (2) Ion spray mass-spectrometry was employed to qualify the variation in amount of naproxen per mole of lysozyme [10]. (3) The charge of the conjugate was compared with that of lysozyme by cation exchange chromatography. Briefly, conjugate or lysozyme was separated on the cation exchange column Mono-S (Pharmacia, Woerden, The Netherlands) using a mobile phase of NaCl (0.2 M) and Tris (10 mM) /HCL (pH 7.2) 0.5 ml/min.

This method was also used for purification of the conjugate and characterization of the iodinated products.

Radiolabeling

For body distribution studies by external counting, ^{123}iodine was introduced in lysozyme with a supposed incorporation in the tyrosine residue of the protein molecule. To ensure radiolabeling of only the naproxen conjugated lysozyme, possible contaminating native lysozyme molecules were removed by separation on a cation exchange column beforehand. The conjugate fraction was dialyzed against water with a Spectra/Por membrane with a 3,500 molecular weight cut off (Spectrum Medical Industries, Houston, Tx, USA) and lyophilized before radiolabeling. Conjugate and native lysozyme were iodinated using the chloramine-T method as described previously [11]. ^{123}Iodine was chosen as radiolabel to allow *in-vivo* imaging by external counting. The efficiency of iodine labeling was high for both compounds: 90 % (conjugate) and 99 % (lysozyme) iodine incorporation. Cation exchange showed no detectable amounts of radiolabeled native lysozyme molecules in the batch of ^{123}I-conjugate.

Protocol for the in-vivo study focusing on the lysozyme moiety

To study the influence of naproxen conjugation on the body distribution of lysozyme, the lysozyme moiety of the conjugate was radiolabeled and followed *in-vivo* by external γ-radiation scanning. Male Wistar rats (250 g) were anesthetized with pentobarbital intraperitonally (60 mg/kg) and placed on a low-energy collimator of a gamma camera. The rats received either 1 MBq of ^{123}I-conjugate mixed with 2 mg of unlabeled conjugate in 0.5 ml of 5% glucose (n = 12) or, as a comparison, equimolar amounts of the free components of the conjugate, i.e. native ^{123}I-lysozyme, lysozyme and naproxen in 0.5 ml 5% glucose (n = 12). The solutions were administered intravenously as a bolus via the dorsal penal vein. The radioactivity was recorded for 120 min with a 1 min resolution.

The radioactivity time course of the right kidney was plotted after analysis of the respective "regions of interest" from the image of the rat on the screen of the gamma-camera (Fig. 2[a]) as previously described [11].

Protocol for the in-vivo study focusing on the naproxen moiety

In a separate group of rats, the influence of lysozyme-conjugation on the renal disposition of naproxen was studied. The amount of naproxen in plasma and kidney was determined at different time intervals after intravenous injection of conjugate or unbound naproxen. Under a light anesthesia of Forene/O_2, male

Wistar rats (250 g) received either 2 mg of conjugate in 0.5 ml 5% glucose or equimolar amounts of lysozyme and naproxen (47 µg) via the dorsal penal vein. At 5, 10, 30, 120 or 240 min after injection, rats (n =3) were anesthetized (Forene/O_2), blood was collected and the kidneys extirpated after being flushed with water. The kidneys were lyophilized and stored together with the plasma at - 20 °C until analysis.

Protocol for the pilot study of in-vivo effect

A female Wistar rat (230 g) was kept on a low-salt diet (0.05% NaCl/20% protein; Hope Farm Inc. Woerden, The Netherlands). One week before the experiment, the jugular vein was cannulated to allow a chronic drug administration intravenously . Throughout the experiment, the rat was housed in a metabolic cage and was given food and tap water ad libitum. The first 3 days of the experiment, the rat was injected with vehicle (5% glucose, 1ml/kg rat) twice a day (at 8:30 a.m and 4:30 p.m). Thereafter, the rat was treated for 3 days with 8.5 mg/kg conjugate (0.25 mg/kg conjugated naproxen) twice a day. Then the dose of conjugate was raised to 85 mg/kg conjugate two times a day. Urine was collected for 24 hours at 4 °C and stored at - 20 °C until analysis. Prostaglandin E_2 concentration in the urine was measured by enzyme immunoassay.

HPLC analysis of naproxen

The average amount of naproxen in the conjugate and the plasma and renal concentrations of naproxen were measured by HPLC, with minor modifications as described by Franssen et al [6]. The HPLC determination of naproxen was performed using a Jasco pump (model 880 PU, Tokyo, Japan) and a Jasco autosampler (model 851-AS). The separation column was a µBondapak C18 (30 cm times 3.9 mm I.D.; Millipore Waters, Milford, MA, USA) guarded with a µBondapak C18 Guard-pak precolumn (Millipore Waters). The HPLC peak integration was performed using the integration software JCL6000 (Jones Chromatography, Littleton, CO, USA). The mobile-phase consisted of water/acetonitrile 1/1 and 1% of acetic acid. The flow rate was 1 ml/min. Naproxen was detected with a fluorescence detector (Jasco model 820 EP), operating at excitation wavelength of 330 nm and emission wavelength of 360 nm. Ketoprofen was used as internal standard and recorded simultaneously at 254 nm (Jasco model 875-UV). The retention time of naproxen and ketoprofen was about 12.6 and 20 min, respectively.

After lyophilization, the soft renal tissue was weighed and pulverized to a homogenous powder. The large renal arteries and veins remained, which

allowed an easy removal of these contaminating parts. Dried renal tissue (20 mg) was added to water (1 ml) and extensively disrupted and homogenized by ultrasonic sound using a Vibra cell (Sonics and Materials, Danbury, CT, USA). Naproxen was determined after an extraction procedure. Plasma (10 µl diluted with water to 100 µl) or 100 µl kidney homogenate was added to 100 µl NaOH 6 N. The extraction was performed after hydrolysis for 48 hr at 80 °C to release naproxen from the conjugate. The samples were subsequently acidified with 150 µl of HCl 6 N. After this, 1 ml of dichloromethane and 0.4 µg ketoprofen (100 µl 4 µg/ml) was added and mixed for 1 min. Of the organic layer, 400 µl was transferred to an autosampler vial and evaporated. The residue was dissolved in 400 µl eluens to make it suitable for HPLC analysis. The fraction of unbound naproxen present in the conjugate and unbound naproxen and naproxen-lysine in plasma was determined by the same procedure without hydrolysis for 48 hr, 80 °C.

Kinetic and statistical analyses

The renal half life and total amount of the naproxen-lysozyme conjugate that was finally taken up in the kidney was calculated from the time-course of radioactivity (protein moiety) or naproxen (drug moiety) in the kidney using a computer program for non-linear curve-fitting, MultiFit (Department of Pharmacokinetics and Drug Delivery, University Center of Pharmacy, Groningen, The Netherlands), using the simplex algorithm [12]. All data are expressed as mean ± SEM, except for the half life of conjugate obtained from the renal tissue concentration time curve of naproxen, for which the standard error is given.

Results

Synthesis and characterization

The reaction of 3 mol of preactivated naproxen with 1 mol lysozyme (Fig.1[a]) resulted in a conjugate with an average coupling degree of 2.0 mol naproxen to 1 mol of lysozyme. The amount of uncoupled naproxen was less than 0.1 %. Qualitative mass analysis showed conjugates with different degrees of coupling: 0, 1, 2, 3 and 4 mol of naproxen to 1 mol of lysozyme (Fig.1[b]). Since coupling of the naproxen molecules masks the positively charged amino groups of lysozyme, a reduced retention of lysozyme on a cation exchange column was anticipated. Indeed a retention of 10 min was found for the conjugate as compared to 20 min for lysozyme (Fig.1[c]). The 2 to 1 mol conjugate used in the

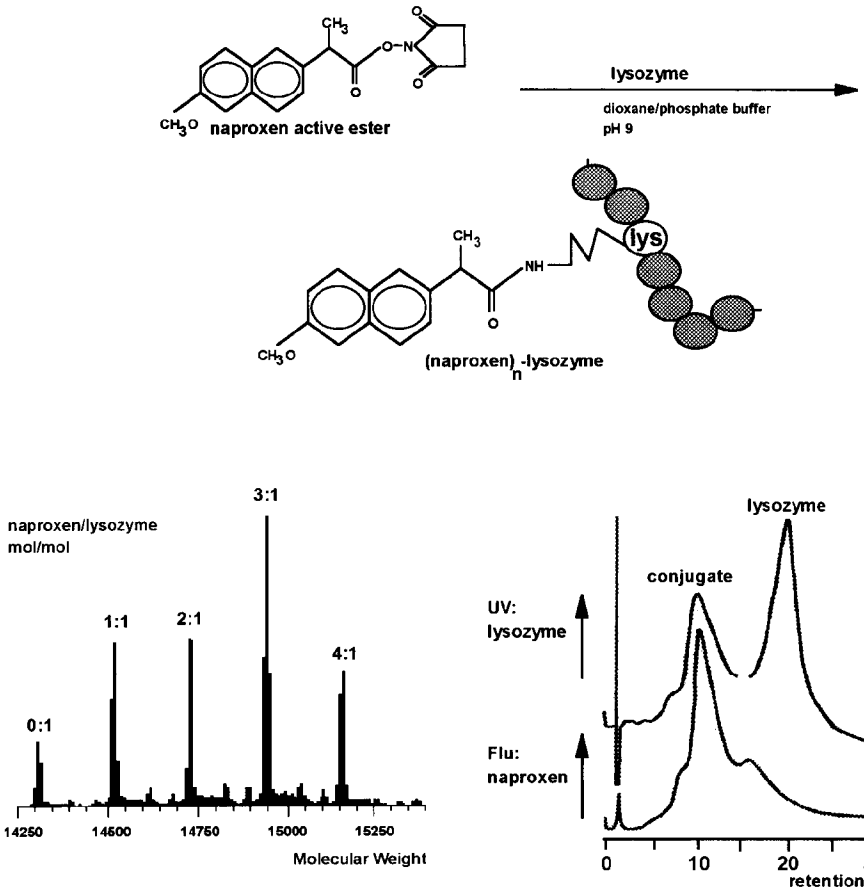

Figure 1. Top: *Synthesis of naproxen-lysozyme conjugate.* Addition of naproxen-N-hydroxysuccinimide to lysozyme results in a naproxen-lysozyme conjugate with a peptide linkage between the free carboxylic group of naproxen and one of the free amino-groups of lysozyme.
Left: *Qualitative distribution of naproxen molecules covalently coupled to lysozyme molecules in the conjugate.* A qualitative measurement by ion spray mass-spectrometry.
Right: E*ffect of naproxen conjugation on the net charge of lysozyme.* Separation of native lysozyme and conjugate was performed on a cation exchange column. The lower solid line represents conjugated naproxen measured by fluorescence detection. The upper line represents conjugated lysozyme (left) and native lysozyme (right), both measured by ultraviolet detection.

present study had a limited solubility in saline but could be dissolved readily in 5% glucose up to 25 mg/ml. A naproxen load of more than 2 to 1 mol lysozyme

seriously reduced the solubility of the conjugate in water as well as in 5% glucose and was disregarded for the present study.

Lysozyme kinetics

The effect of naproxen conjugation on the renal uptake and degradation of lysozyme was studied in the rat by external γ-radiation counting. Qualitative analysis showed a striking similarity in the body distribution of native lysozyme and conjugate (figure 2[a]).

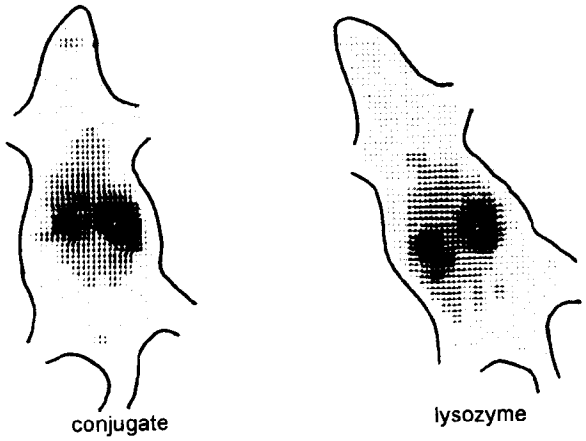

Figure 2[a]: *Body distribution of naproxen-^{123}I-lysozyme conjugate and native ^{123}I-lysozyme.* Representative images, with a 1 min resolution, about 20 min after an intravenous injection of naproxen-^{123}I-lysozyme conjugate (left) and native ^{123}I-lysozyme (right) in the rat.

Comparison of the concentration time-course of conjugate and native lysozyme in the kidney (Fig. 2[b]) revealed that conjugation did not affect the renal handling of lysozyme. Both proteins were rapidly taken up by the kidneys, with a maximum content of 1360 ± 140 counts per minutes (conjugate) and 1240 ± 100 counts per minutes of radioactivity (lysozyme) at 18 min after injection. Quantification with a kinetic analysis of the radioactivity in the kidneys as described previously [11], revealed that 68 ± 4 % (conjugate) and

67 ± 3 % (lysozyme) of the injected dose was finally taken up by the kidneys. Eighteen minutes after injection, the apparent intrarenal rate of degradation of both proteins exceeded the rate of uptake as indicated by the subsequent gradual decrease in renal radioactivity. The disappearance of the tissue radioactivity half life appeared to be in the same order for conjugate (283 ± 40 min) and native lysozyme (228 ± 26 min) ($p < 0.05$).

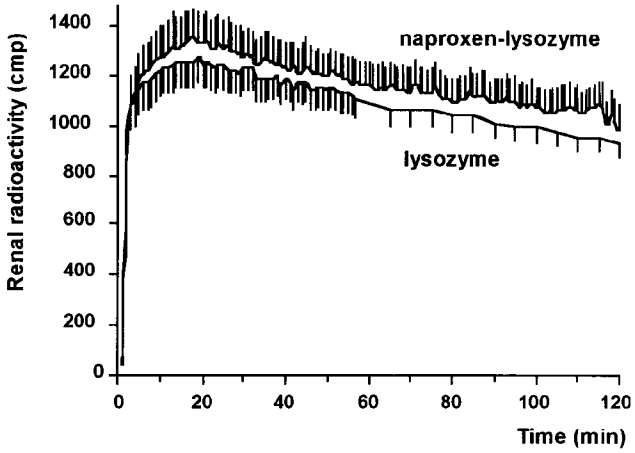

Figure 2[b]: *Renal time-course of naproxen-^{123}I-lysozyme conjugate and native ^{123}I-lysozyme after an intravenous injection in 12 rats.* Values are given as mean + SEM for conjugate and mean - SEM for native lysozyme.

Naproxen kinetics

Conjugation of naproxen and lysozyme resulted in an increased plasma clearance of naproxen compared to unbound naproxen (Fig.3). Since a reduction in extrarenal side effects is one objective of drug targeting, it was encouraging to establish that no detectable amount of free naproxen was present in the plasma after administration of conjugate.

As shown in figure 4, conjugation to lysozyme resulted in a pronounced increase of naproxen accumulation in the kidney (6.19 ± 0.5 and 0.09 ± 0.03 µg/kidney at 30 min after injection for conjugated and unbound naproxen, respectively). In agreement with the gamma camera studies, the naproxen-conjugate was rapidly taken up by the kidneys and subsequently degraded, as indicated by the gradual lowering of the total naproxen tissue content with time. The rate at which naproxen and potential metabolites left the kidney was significantly faster ($t½$ = 156 ± 28 min) than that of the radiolabeled breakdown products derived from the lysozyme-moiety of the conjugate ($t½$ = 283 ± 40 min) ($p < 0.05$).

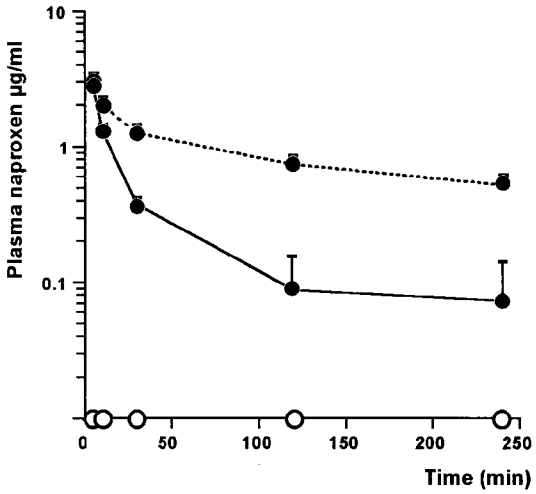

Figure 3. *Plasma naproxen as a function of time after an intravenous injection of parent naproxen and conjugated naproxen in three rats at each indicated time point.* Plasma disappearance of naproxen after injection of parent naproxen (dotted line, closed circles) and plasma disappearance of conjugated naproxen after injection of conjugate (solid line, closed circle). Plasma concentration of parent naproxen after injection of naproxen-lysozyme conjugate (open circles). Values are given as + SEM.

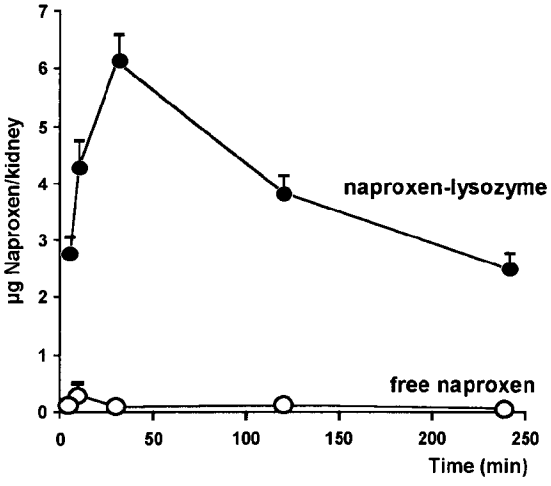

Figure 4. *Renal time course of naproxen after an intravenous injection of parent naproxen or conjugate in three rats at each indicated time point.* The line with open circles represent the parent naproxen after injection of parent naproxen. The line with closed circles represents conjugated naproxen after injection of conjugate. Values are given as + SEM.

Urinary excretion of prostaglandin E_2

This preliminary study shows that a daily treatment of 0.5 mg/kg conjugated naproxen did not reduce urinary excretion of prostaglandin E_2 (PGE_2). However, during an intravenous treatment of 5 mg/kg/day conjugated naproxen, PGE_2 excretion was reduced by 50% (Fig. 5).

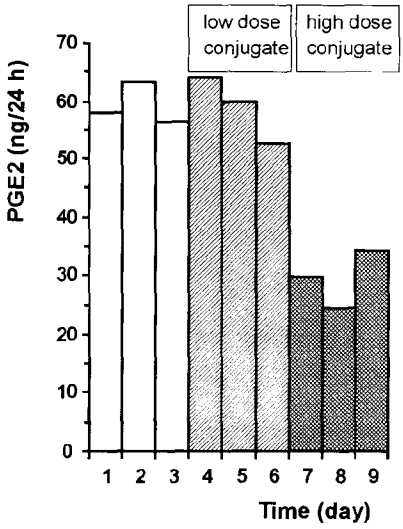

Figure 5. *Urinary excretion of prostaglandin E_2 after daily intravenous injection of vehicle, low dose conjugate and high dose of conjugate, respectively.* A typical example of a rat treated with vehicle (days 1-3), with 0.5 mg/kg/day conjugated naproxen (days 4-6) and with 5 mg/kg/day conjugated naproxen (days 7-9).

Discussion

In the present study, a well defined naproxen-lysozyme conjugate with an average coupling degree of 2 mol of naproxen to 1 mol of lysozyme was studied *in-vivo* with emphasis on the renal kinetics of both the lysozyme and naproxen-moiety of the conjugate. The data show that renal specific targeting of naproxen is indeed obtained by conjugation of the drug to lysozyme.

In agreement with previous studies [11,13], a significant amount of intravenously injected native lysozyme rapidly accumulated in the kidneys with a subsequent slowing of intrarenal degradation. Conjugation of lysozyme with the drug naproxen did not affect these characteristics of renal delivery and breakdown of the LMWP. Thus, in spite of conjugation, lysozyme remains renal

specific and accessible to renal degradation and is therefore suitable to serve as a renal specific carrier of naproxen. In contrast, focusing on the drug moiety of the conjugate, conjugation of naproxen using lysozyme distinctly altered the kinetics of the drug. The plasma disappearance of intravenously injected conjugated and unbound naproxen were largely different, with a more rapid disappearance of conjugated naproxen from the circulation. In the kidney, naproxen accumulated about 70 times more if the drug was administered in the conjugated form. Until now, no other renal targeting strategy has shown such a high relative enrichment of a drug in the kidney [14].

The apparent renal accumulation of conjugated naproxen, measured directly by analyzing the dissected kidney, was about two times lower than that found in the gamma-radiation lysozyme experiment. Extrapolation of the renal tissue concentration time curve to time of injection revealed that about 30% of conjugated naproxen was taken up by the kidneys, while the lysozyme gamma-imaging study indicated that 70% of the injected dose entered the renal compartment. One possible explanation for this discrepancy may be that iodine and naproxen were not equally distributed over the conjugate molecules. As a result, the iodine kinetics we presented may not fully represent the kinetics of all conjugate molecules, but may reflect only part of conjugate with a certain load of naproxen. Second, the calibration procedure of the gamma-camera may be partly incorrect. Lastly, we may underestimate the renal concentration of total naproxen, for example, due to an incomplete extraction of naproxen from the tissue. In spite of this as yet unresolved discrepancy, the 70 times difference in renal accumulation of naproxen after injection of free and conjugated naproxen clearly indicates a successful renal selective targeting of the drug.

The rate of degradation of the naproxen-lysozyme conjugate in the kidney as calculated on the basis of the time-course of renal ^{123}iodine activity was lower than that of the time-course of naproxen content in the kidney. This difference may be explained by an unequal accessibility of the protein backbone contacting the two labels by the proteolytic enzymes in the lysosomes of the proximal tubular cell or by a change in affinity for particular degradation enzymes as the consequence of iodine or naproxen labeling. Alternatively, the difference may be explained by an unequal rate of renal release of the two breakdown products.

In an earlier study [6], it was suggested that a part of naproxen-lysozyme conjugate is liberated in the kidney as naproxen-lysine and that this active metabolite is at least partly excreted to the blood, finally to be excreted in the gut. However, in that particular study, taken into account that the naproxen content in the kidney was not measured, the plasma levels of naproxen-lysine

were undetectable and the solubility of the conjugate was rather poor, it can not be excluded that the naproxen-lysine in the gut originating from conjugate was extracted by the liver. In contrast, in the present study, a renal selective uptake of conjugated naproxen was proven by the detection of naproxen in the kidney.

The present study is the first preliminary report of a renal effect of naproxen-lysozyme conjugate. Treatment with the conjugate resulted in a reduced urinary excretion of prostaglandin E_2. Together with the fact that no detectable amounts of free naproxen or active metabolite were found in the circulation, it seems reasonable to conclude that a renal specific effect of naproxen was obtained successfully by coupling the drug to lysozyme. Since the ultimate goal of renal targeting of naproxen is the treatment of proteinuria, the question may arise as to whether the proximal tubular cell is an appropriate site of naproxen release and whether a reduced PGE_2 excretion is an indication that the conjugate will be effective in reducing proteinuria. In patients with the nephrotic syndrome, the degree of reduction of proteinuria by NSAID treatment correlates with the degree of reduction of urine prostaglandin E_2 excretion [15]. However, little is known about the mechanism by which NSAIDs reduce proteinuria. Glomerular and vascular prostaglandins are directly involved in the hemodynamic regulation while the tubular and interstitial prostaglandins indirectly regulate the hemodynamics by control of homeostasis and hormonal stimulation [16]. Since naproxen released from the proximal tubular cell may only reach its cyclooxygenase targets downstream and not those of the glomerulus, the use of the naproxen-lysozyme conjugate may provide an opportunity to study the direct and indirect involvement of prostaglandins in the regulation of hemodynamics and proteinuria.

In summary, renal specific targeting of the NSAID naproxen can be obtained by conjugation with the LMWP lysozyme. This conjugate is renal selective and biodegradable, while after renal uptake and degradation of the conjugate free naproxen is not detected in the systemic circulation. This renal targeting concept may provide the opportunity to improve the therapy of kidney diseases and to increase the knowledge of renal pharmacology. The concept may be further elaborated by the use of other types of renal-specific drug carriers such as tailor-made synthetic polymers or by the coupling of other kind of drugs such as angiotensin converting enzyme (ACE)-inhibitors and anti-inflammatory steroids. Also, selective targeting to other renal compartments such as glomerular endothelial and mesangial cells are attractive options for further study.

Acknowledgment

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Chapter 6

Renal targeting of a NSAID: effects on renal prostaglandins

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Submitted to Clinical Science

Abstract

To achieve renal specific targeting of the NSAID naproxen, the low-molecular-weight protein lysozyme was employed as carrier since it is mainly taken up and catabolized in the lysosomes of the proximal tubular cell of the kidney. A previous study showed that conjugation to lysozyme resulted in a 70-fold increase of naproxen accumulation in the kidney with a subsequent renal release of the active metabolite naproxen-lysine. In the present study we questioned whether naproxen-lysozyme is active in the kidney inhibiting the urinary excretion of prostaglandin E_2 (PGE_2) and renal sodium and water excretion related to prostaglandin inhibition. The effects were measured in salt restricted baseline condition as well as during furosemide treatment in the rat. A high dose of 10 mg/kg/day free naproxen did not affect PGE_2 excretion in baseline condition (naproxen 11 $\pm$ 1 ng/8h versus vehicle 13 $\pm$ 4 ng/8h) whereas sodium and water excretion were respectively 3.0 and 1.6 times lower in the naproxen group ($p < 0.05$). Naproxen completely prevented the furosemide induced increase (3-fold) in PGE_2 excretion in the vehicle group (naproxen 6.6 $\pm$ 1.1 ng/8h, vehicle 40 $\pm$ 12 ng/8h, $p < 0.005$). Furosemide stimulated natriuresis was 1.6 ($p < 0.05$) and diuresis 1.8 times ($p < 0.005$) lower in the naproxen group.

A dose of 2 mg/kg/day lysozyme conjugated naproxen did not affect PGE_2 excretion in baseline condition (conjugate 18 $\pm$ 2 ng/8h, vehicle 24 $\pm$ 5 ng/8h). The conjugate also had no effect on sodium and water excretion. However, the naproxen conjugate completely prevented furosemide induced increase (2-fold) in PGE_2 excretion in the vehicle group (conjugate 16 $\pm$ 3 ng/8h, vehicle 48 $\pm$ 13 ng/8h, $p < 0.05$). Surprisingly, furosemide induced natriuresis and diuresis were not affected by the conjugate. In conclusion, a renal specific delivery of the NSAID naproxen using lysozyme results in an inhibitory effect on renal PGE_2 synthesis but does not affect the excretion of sodium and water. With regard to the latter aspect, the effect of the naproxen-lysozyme conjugate differs from a high dose of free naproxen.

Introduction

Drug targeting is defined as the technology to direct a therapeutic agent specifically to the desired site of action for site-specific activity with little or no interaction with non-target tissue. Several approaches of drug targeting to the kidney have been studied, focusing on delivery of several kind of drugs to several sites of the nephron [1].

We proposed drug targeting to the proximal tubular cell of the kidney using a low-molecular-weight protein (LMWP) as carrier as reviewed by Franssen et al. [2]. LMWPs are rather freely filtered by the kidneys and after reabsorption in the proximal tubular cell, catabolized in the lysosomes [3-5]. Thus, drugs attached to a LMWP may be carried to the kidney without affecting other tissues. After renal release they may then induce a specific renal effect. We recently showed that the non-steroidal anti-inflammatory drug (NSAID) naproxen could be targeted to the kidney by coupling it to the LMWP lysozyme [6]. Conjugation to lysozyme resulted in a 70-fold increase of naproxen accumulation in the kidney with a subsequent gradual reduction of renal drug content in time [7]. Of note, not the parent drug naproxen was intrarenally released from the conjugate but the more hydrophilic metabolite naproxen-lysine. This metabolite appeared to be as active as parent naproxen in inhibiting prostaglandin synthesis in-vitro [6].

Because of the promising kinetic features, we questioned whether naproxen-lysozyme conjugate is indeed effective in the kidney in-vivo. Naproxen is an inhibitor of cyclo-oxygenase, blocking the synthesis of prostaglandins. Among others, it reduces the urinary excretion of prostaglandin E_2 (PGE_2) which reflects renal PGE_2 synthesis [8-13].

Our primary objective in this study was to test whether the naproxen-lysozyme conjugate could inhibit the urinary excretion of PGE_2. Our secondary objective was to study whether the conjugate has any effects on renal sodium and water excretion related to prostaglandin inhibition.

Under physiological condition, renal prostaglandin production is low and not essential to control natriuresis and diuresis as revealed by the inability of NSAIDs to affect water and salt excretion [14,15]. However, sodium depletion as well as pharmacological intervention with e.g. furosemide are well known stimuli of PGE_2 production and enhance PGE_2 involvement in sodium and water homeostasis [8,9,16-21]. Therefore, we tested the effect of naproxen-lysozyme conjugate on renal PGE_2 excretion in baseline sodium restricted condition as well as during furosemide treatment in the rat. In addition, the effect of the

conjugate on sodium and water excretion was measured in these two renal prostaglandin stimulated conditions.

Materials and Methods

Chemicals

Lysozyme, the sodium salt of naproxen and indomethacin were purchased from Sigma (Axel, The Netherlands). Naproxen-lysozyme conjugate was synthesized as described previously [7]. Briefly, naproxen was activated with N-hydroxysuccinimide [22]. Naproxen-N-hydroxysuccinimide 10.5 mg (30 µmol) in 6 ml dioxane was added in a drop-wise fashion to 140 mg (10 µmol) lysozyme in 24 ml (50 mM) Na_2HPO_4 and stirred for 2 hours. After centrifugation (2000 r.p.m, 10 min), the product was purified using a Sephadex G-25 column (Pharmacia, Woerden, The Netherlands) with dioxane/water 80/20 vol/vol as mobile phase. The protein fraction of the column was washed with water and concentrated in an Amicon stirred cell concentrator using an YM3 membrane (Amicon, Beverly, MA, USA). After lyophilization, the conjugate was stored at - 20 °C. The coupling degree of the conjugate used in the present study was an average of 1.7 mol naproxen to 1 mol lysozyme.

Instrumentation

Male Wistar rats (270 g) were kept on a low-salt diet (0.05% NaCl/20% protein, Hope Farm Inc. Woerden, The Netherlands) throughout the study. One week before the experiment, the jugular vein was cannulated to allow intravenous drug administration [23]. During the experiment, blood clotting in the jugular cannula was prevented by filling the cannula with 75 µl heparine (50 I.E./ml) after each drug administration. The rats were housed individually in metabolic cages in a temperature controlled room with a 12 hour light/dark cycle.

Protocol of the study

First, the effect of a high dose naproxen [9,12,13] on PGE_2 excretion, natriuresis and diuresis was established in sodium depleted rats (baseline period). For 3 days, rats received intravenously either a high dose of 10 mg/kg naproxen (n = 6) or the vehicle used for dissolution of naproxen (glucose 5%, 2 ml/kg body weight, n = 6) once a day, at 8:30 a.m. Subsequently, the effect of naproxen on furosemide stimulated PGE_2 excretion, natriuresis and diuresis

was established. For that purpose the same naproxen or vehicle treated rats received furosemide (10 mg/kg) subcutaneously on day 4 and 5 at 9:30 a.m.
Second, the effect of conjugate treatment on PGE_2 excretion, natriuresis and diuresis was established in sodium depleted rats (baseline condition). The conjugate was administered two times a day to obtain a rather stable renal level of naproxen throughout the day. For 3 days, rats received intravenously either a dose of 1 mg/kg lysozyme conjugated naproxen (n = 6) or vehicle (glucose 5% 2 ml/kg body weight, n = 6) twice a day, at 8:30 a.m and 4:30 p.m. Subsequently, the effect of the conjugate on furosemide stimulated PGE_2 excretion, natriuresis and diuresis was established in the same rats. For that purpose the same conjugate and vehicle treated rats received furosemide (10 mg/kg) subcutaneously on day 4 and 5 at 9:30 a.m. Finally, the same protocol was performed with the two components of the conjugate in their free forms, separately. Free naproxen (1 mg/kg) and native lysozyme (34 mg/kg) in equimolar dose as conjugate were intravenously injected twice a day, at 8:30 a.m and 4:30 p.m (each group, n = 6). On day 4 and 5 at 9:30 a.m, furosemide (10 mg/kg) was given additionally to all rats subcutaneously.

Measurements
Urine was collected daily for 8 hours (from 8:30 a.m to 4:30 p.m) at 4 °C with indomethacin (50 µl 10 mg/ml ethanol) to prevent possible breakdown and/or ongoing synthesis of PGE_2, respectively. Urine was weighed with an accuracy of 0.02 gram and stored at - 20 °C until analysis. Body weight and intake of water and food was measured daily at 8:30 a.m with an accuracy of 2 gram. Urine prostaglandin E_2 (PGE_2) concentration was determined by enzyme immuno assay system (Amersham Life Science, 's-Hertogenbosch, The Netherlands). Urine sodium concentration was measured by flame photometry (Perkin Elmer 3030B Atomic Absorption Spectrophotometer).

Data analysis
Results are presented as means ± SEM. All comparisons were made using the non-parametric Mann-Whitney test. P values of less than 0.05 were considered statistically significant.

Results

In the baseline (low salt) condition, body weight and sodium and water intake were the same in all groups of rats receiving different kinds of treatments. Also

during furosemide, body weight as well as sodium and water intake were not different between the conjugate, naproxen, lysozyme and vehicle treated animals (table 1). These data indicate that all treatments were tolerated without affecting appetite and growth of the rat differently in both the baseline and the furosemide-treated condition. Comparison between the baseline and furosemide-treated condition revealed a difference in water intake. Water intake was higher in all groups on day 4 and 5 (during furosemide) as compared to day 2 and 3.

Tabel 1.

treatment	furosemide 10 mg/kg/day	Body weight (g)	sodium intake (µmol/24 h)	water intake (ml/24 h)
vehicle 1	-	292 ± 11	151 ± 11	27 ± 2
naproxen (10 mg/kg/day)	-	284 ± 9	153 ± 6	26 ± 3
vehicle 2	-	300 ± 14	157 ± 12	19 ± 2
conjugate (2 mg naproxen/kg/day)	-	304 ± 10	185 ± 6	20 ± 1
naproxen (2 mg/kg/day)	-	298 ± 8	172 ± 27	21 ± 2
lysozyme (68 mg/kg/day)	-	295 ± 13	164 ± 14	20 ± 2
vehicle 1	+	293 ± 10	140 ± 12	59 ± 2
naproxen (10 mg/kg/day)	+	287 ± 7	147 ± 10	39 ± 5
vehicle 2	+	287 ± 14	114 ± 23	35 ± 5
conjugate (2 mg naproxen/kg/day)	+	303 ± 10	133 ± 17	36 ± 1
naproxen (2 mg/kg/day)	+	298 ± 11	140 ± 23	39 ± 5
lysozyme (68 mg/kg/day)	+	290 ± 15	103 ± 26	34 ± 7

- furosemide: mean ± SEM of day 2 and 3 (n = 6).
+ furosemide: mean ± SEM of day 4 and 5 (n = 6).

Unbound naproxen

First, we studied whether a high dose of free naproxen inhibited PGE_2, sodium and water excretion in the baseline (low salt) condition. As shown in figure 1, PGE_2 excretion in baseline condition was not different between vehicle and naproxen treated rats. In contrast, natriuresis and diuresis were respectively 3.0 and 1.6 times lower in the naproxen group ($p < 0.05$). Second, we tested the effect of the high dose of naproxen on the furosemide response (figure 1, day 4 and 5). Furosemide induced a 3-fold increase in PGE_2 excretion in the vehicle treated rats. The high dose of naproxen completely prevented this furosemide induced rise in PGE_2 excretion ($p < 0.005$, day 4). In spite of this pronounced PGE_2 effect, the furosemide stimulated natriuresis was only 1.6 times ($p < 0.05$, day 5) and diuresis 1.8 times ($p < 0.005$, day 5) lower in the naproxen group compared to the vehicle group.

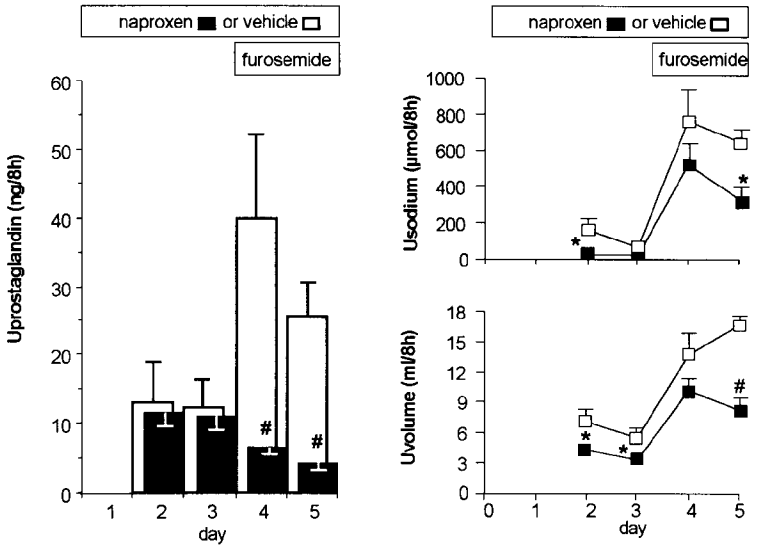

Figure 1. *Urinary excretion of PGE_2, sodium and water in vehicle and high dose naproxen treated rats.* Rats were treated with vehicle (white bars, white squares) or 10 mg/kg naproxen (black bars, black squares) daily. Furosemide 10 mg/kg was given on day 4 and 5 additionally. Urine parameters were determined in urine collected during day-time. Data are expressed as mean + SEM (n = 6). Difference of naproxen versus vehicle treated rats: * $p < 0.05$ and # $p < 0.005$.

Conjugate

The conjugate had no effect on PGE_2 excretion in the baseline (salt depleted) condition when compared to vehicle as shown in figure 2. The conjugate also had no effect on sodium and water excretion during baseline (figure 2). As observed in the above described experiments, furosemide stimulated PGE_2 excretion in the vehicle treated group (figure 2, day 4 and 5). The conjugate fully prevented this furosemide induced rise in PGE_2 excretion ($p = 0.04$ on day 4). Interestingly, despite this effect on renal prostaglandin synthesis, furosemide induced natriuresis and diuresis were not affected by the conjugate.

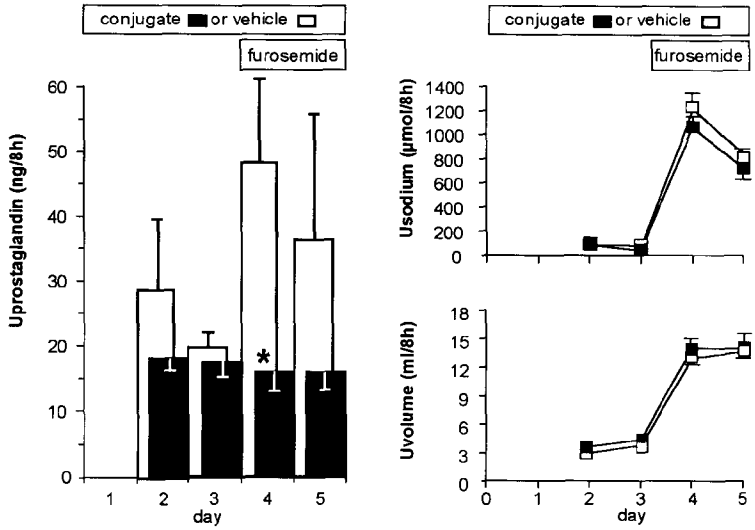

Figure 2. *Urinary excretion of PGE_2 , sodium and water in rats treated with vehicle or naproxen-lysozyme conjugate.* Rats were treated with vehicle (white bars, white squares) or 34 mg/kg naproxen (black bars, black squares) twice a day. Furosemide 10 mg/kg was given on day 4 and 5 additionally. Urine parameters were determined in urine collected during day-time. Data are expressed as mean + SEM (n = 6). Difference of conjugate versus vehicle treated rats: * $p < 0.05$.

Control compounds

Lastly, we studied the effect of the two components of the conjugate in their free form, separately, e.g. native lysozyme and free naproxen in an equimolar dose as conjugate. Both free components had no effect on PGE_2 excretion in the salt depleted rat when compared to vehicle. The components neither had an effect on sodium and water excretion in salt depleted condition. In furosemide stimulated condition, neither any detectable differences were found in PGE_2 excretion and sodium and water excretion in the low dose free naproxen and lysozyme groups compared to the vehicle group.

Discussion

The non-steroidal anti-inflammatory drug (NSAID) naproxen was coupled to the low-molecular-weight protein (LMWP) lysozyme to deliver the drug specifically to the proximal tubular cell of the kidney where it may regain

NSAID activity after the intracellular degradation of the drug-LMWP conjugate. The present study demonstrates that the naproxen-lysozyme conjugate is indeed active in the kidney. Just like free naproxen, it prevents furosemide induced excretion of PGE_2. Surprisingly, the effect occurred in the absence of a change in diuretic and natriuretic response to furosemide in which it differs from treatment with a high dose of free naproxen.

Before testing the conjugate, we studied whether a high dose of free naproxen inhibited PGE_2, sodium and water excretion in our rat model. The design of a reliable animal model to study NSAID activity is complicated by the complexity and incomplete knowledge of the prostaglandin system in the control of renal function [14,15]. The synthesis of renal prostaglandins as well as the prostaglandin dependency of renal function is highly sensitive for multiple factors like volume depletion, anesthesia, surgery and salt intake. Because of that, subtle differences in experimental condition are probably responsible for the large variations in outcome found in the literature. We chose to study the renal effects of naproxen during low salt intake as well as during furosemide stimulation. In salt restricted condition, a high dose of naproxen did not affect PGE_2 excretion whereas natriuresis and diuresis were significantly lowered. The discrepancy may indicate that prostaglandin-unrelated actions of naproxen are involved in affecting sodium and water excretion in low salt state [15]. Second, it could be that a moderate change in PGE_2 excretion occurred that was too small to detect.
The effect of furosemide on PGE_2 excretion was very clear and a high dose of naproxen was able to prevent this effect, which is in line with other studies [8,9,21,24]. Despite the complete blockade of furosemide induced PGE_2 excretion, the furosemide induced natriuresis and diuresis were only partially reduced by the high dose of naproxen.

After having confirmed that at least the furosemide approach was suitable to measure NSAID activity, the naproxen-lysozyme conjugate was tested. In this respect it should be realized that the amount and time-course of active compound in the renal tissue will be quite different after treatment of conjugate and free naproxen: after intravenous injection of free naproxen, the active drug concentration in the body is instantly high and is reduced in time with a half-life of 6 hours [25]. After injection of conjugate, no active compound is present at start but is gradually formed in the kidney with a formation half-life of 2.5 hours [7]. The active metabolite of the conjugate, naproxen-lysine is released from the lysosomes of the proximal tubular cell and may distribute within the kidney

and be eliminated from the kidney in a different pattern compared with the more hydrophobic drug naproxen that is secreted via the proximal tubular cell. The results of the present study may reveal some of the kinetics and dynamics of targeted and untargeted NSAIDs in the kidney.

Did treatment of the rats with targeted naproxen induce renal NSAID activity? Administration of the naproxen-lysozyme conjugate did not affect PGE_2 excretion in the baseline salt-depleted animals similar to the high dose of free naproxen. In contrast with the high dose of free naproxen, natriuresis and diuresis were neither affected by the conjugate. Potential explanations for these data are: 1) the amount of active metabolite that was generated in the kidney was too low to produce significant effects, 2) the metabolite did not reach the renal sites of prostaglandin synthesis that are responsible for sodium and water excretion or 3) the metabolite does not exhibit prostaglandin-unrelated actions affecting sodium and water excretion in contrast with parent naproxen [15].
A more clear picture arose from the furosemide stimulated condition, since in that condition the high dose of free naproxen affected natriuresis and diuresis as well as PGE_2 excretion. The furosemide induced PGE_2 excretion was completely abolished in the conjugate treated animals. This indicates that the active metabolite of the conjugate, naproxen-lysine reached renal sites of PGE_2 synthesis. However, the furosemide induced natriuresis and diuresis were not affected by conjugate. Either, the metabolite does not have the prostaglandin-unrelated actions on sodium and water excretion or the active metabolite of the conjugate followed an other intrarenal pathway than the parent naproxen. With respect to the latter explanation, different kind of prostaglandins are synthesized along the nephron [14,15,26]. The contribution of the different prostaglandins formed at the different sites of the nephron in the regulation of sodium and water excretion is complex and depends on many factors including the sodium state and kind of intervention [26]. Our data may imply that the active metabolite of the conjugate reached renal sites of PGE_2 synthesis sufficiently but did not reach those sites where the prostaglandins are synthesized that are involved in furosemide induced natriuresis and diuresis. Free naproxen is extensively reabsorbed in the distal tubule of the kidney [27] via which route it may effectively inhibit prostaglandin synthesis in the medullary interstitial cells. On the other hand, the active metabolite of the conjugate, naproxen-lysine is more hydrophilic and may not reach those sites of prostaglandin synthesis that are involved in furosemide induced sodium and water excretion [26].

Does conjugation to lysozyme increases the renal potency of naproxen? Naproxen in the conjugated form was administered in a 5 times lower dose than the high dose free naproxen but produced similar effects on PGE_2 excretion. Conjugation to lysozyme likely increased the renal accumulation of (yet inactive) naproxen and, through release of active naproxen-lysine may have increased the renal naproxen activity as well [7]. As discussed above, the conjugate completely prevented furosemide induced PGE_2 excretion. Since an equimolar dose of free naproxen (2 mg/kg/day) did not affect furosemide induced PGE_2, we may conclude that conjugation to lysozyme indeed increased the renal accumulation of naproxen and/or increased the intrinsic potency of naproxen in the kidney.

Could the naproxen-lysozyme conjugate be of importance in the clinical situation? In the present study we focused on a number of renal physiological parameters in healthy rats. This because abundant information is available about the renal effects, kinetics and concentrations of NSAIDs under these conditions. In contrast, data of NSAID treatment in animal models of renal disease as well as the effect on pathological parameters is scarce [28-31].
The ultimate goal of renal targeting however is the treatment of disease such as glomerular proteinuria [32,33] and tubular defects such as Fanconi syndrome and Bartter's syndrome [34]. These disorders are primarily located in the glomerulus and proximal tubule and are characterized by an abnormal high synthesis of prostaglandins in the kidney [32,34]. In the present study using healthy animals, it was essential to make the kidney "prostaglandin dependent". In renal disease this will not be necessary: prostaglandin production is highly stimulated and thus the effects of naproxen and conjugate could be more pronounced. The present results indicate that the active metabolite of the conjugate did not reach all sites of prostaglandin synthesis, that is if the effects on sodium and water excretion are taken into account. Yet, glomerular and proximal tubular sites of prostaglandin synthesis are likely the targets of prostaglandin synthesis that should be blocked during disease. It remains to be established whether these sites are reached using the conjugate.

In conclusion, a specific delivery of the NSAID naproxen to the proximal tubular cell of the kidney did produce a clear inhibitory effect on furosemide induced renal PGE_2 synthesis but did not affect the excretion of sodium and water, in this aspect differing from free naproxen. Whether the conjugate is effective in the experimental disease state will be the next objective to study.

Acknowledgment

The student Pharmacy, Folgert Haverdings and medical students, Marieke Vloedbeld, Wouter F.M. Goldschmidt and Lodewijk de Groot are gratefully acknowledged for their contribution in the study. Nienke Koorn, Alex Kluppel and Jacko J. Duker are thanked for their technical assistance. This work was financially supported by the Dutch Kidney Foundation (NSN), grant C 91.1176.

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Chapter 7

Drug targeting to the urinary bladder: an increased urinary excretion and reduced renal toxicity of doxorubicin in the rat

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Abstract

Drug targeting of anti-tumor drugs to the urinary bladder may be of clinical importance since they have a low degree of urinary excretion and/or are highly toxic elsewhere in the body while for the treatment of bladder carcinoma high local concentrations are required. To achieve distal urinary tract targeting of the antitumor drug doxorubicin, it was coupled via the acid-labile *cis*-aconityl linker to the low-molecular-weight protein (LMWP) lysozyme. The coupling procedure was designed to obtain a drug-LMWP conjugate that would still be filtered in the kidney, while at the same time the normal tubular reabsorption of the protein should be reduced, thus allowing efficient excretion of the conjugate into the primary urine. The acid-labile aconityl linker was used to allow the regeneration of the active doxorubicin during the presence of the conjugate in acidified urine, an aspect that is subject for subsequent studies. In the present study we investigated the potential shift in elimination route at normal urine pH range.

The conjugate was tested in rats for its target specificity and general toxicity. Wistar rats were injected intravenously with either a single dose of 2 mg/kg free doxorubicin, a single dose of 2 mg/kg lysozyme-conjugated doxorubicin or a single dose of 10 mg/kg lysozyme-conjugated doxorubicin.

Total urinary excretion of doxorubicin was considerably higher if the drug was coupled to lysozyme : 27 ± 1% and 39 ± 3 % of the given dose was found in the urine after administration of respectively 2 mg/kg and 10 mg/kg lysozyme conjugated doxorubicin versus 4.4 ± 0.4% in case of free doxorubicin. As expected the main part of the doxorubicin excreted in urine after conjugate administration was still protein bound since the urine was not acidified in the present study.

As far as toxicity is concerned, free doxorubicin induced typical renal pathology reflected by a proteinuria of 423 ± 64 mg/24 hours and glomerulosclerosis. None of the rats injected with doxorubicin-lysozyme conjugate showed any of such toxicity (14 ± 3 mg protein/day, no sclerosis). No detectable abnormalities in heart, liver and lung were induced by free and conjugated doxorubicin.

In conclusion, an increased urinary excretion and reduced renal toxicity of doxorubicin is obtained by coupling it to the LMWP lysozyme via an acid-labile linker. This procedure of delivery of an antitumor drug after parenteral administration to the distal urinary tract could be applied in the treatment of bladder carcinoma as an alternative to local administration.

Introduction

Drug targeting, the specific delivery of a drug to a target tissue, is a strategy designed to improve the therapeutic index of a drug [1-3]. For treatment of cancer in the urinary bladder, local drug administration is commonly employed but is far from ideal [4,5]. Local administration allows therapy with drugs such as anti-cancer drugs that, if administered systemically, would reach only sub-therapeutic concentrations in the urinary bladder and/or that are highly toxic elsewhere in the body. We developed a concept of targeting to the urinary tract using systemic administration of a drug-protein conjugate that is predominantly extracted by the kidney with potential regeneration of the active drug in acidified urine.
Low-molecular-weight proteins (LMWPs) may be suitable drug carriers for delivery to the distal urinary tract. LMWPs are rapidly and predominantly cleared by the kidney [6-8] and renal specificity remains after drug attachment [9-11]. However, in physiological circumstances LMWPs are filtered in the glomerulus and subsequently after reabsorption by the proximal tubular cell, degraded in the lysosomes [6,12]. Using LMWPs for drug delivery to the distal urinary tract, tubular reabsorption should therefore be prevented. Reduction of tubular reabsorption can in principle be achieved by co-administration of competing LMWPs as well as certain amino-acids [13-15]. More effectively, tubular reabsorption may be prevented by modification of the molecular structure of the drug-LMWP conjugate itself. For the process of tubular endocytosis, the positively charged free amino-groups of the protein are important [16-18] while for the preparation of drug-protein conjugates, these free amino-groups are used for drug-attachment. Thus, for targeting of LMWPs to the urinary tract, we considered the possibility to react all of the available free amino-groups of the LMWP with drug molecules to prevent the tubular uptake process.
After arrival of the intact drug-LMWP conjugate in the urinary bladder, the drug should be released from the conjugate to regain its pharmacological activity. For this purpose, we interposed an acid-labile spacer between the drug and the LMWP [19] which can be cleaved in the bladder in case the urine is acidified.

In the present study, doxorubicin was used as a model drug. Doxorubicin is effective in the treatment of urinary bladder carcinoma [4,5] but has a low degree of urinary excretion [20,21] and shows severe systemic side-effects [22]. As drug carrier, the LMWP lysozyme was chosen since there is abundant

information about this LMWP as drug carrier to the kidney [9-11]. The acid-labile bond, *cis*-aconityl linker was interposed between the drug and the carrier. We compared the urinary kinetics of administered unbound and conjugated doxorubicin in healthy rats to determine whether a shift in excretion route in favor of the urine was obtained by conjugation. Along with this aspect, we studied the toxicity of doxorubicin in various tissues with special reference to the kidneys which is the primary toxic finding of doxorubicin in the rat.

Materials and Methods

Materials

Doxorubicin.HCL (2 mg/ml injection solution) was obtained from Farmitalia Carlo Erba (Brussels, Belgium). Lysozyme and *cis*-aconitic anhydride was purchased from Sigma (Axel, Netherlands) and EDCI (1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide.HCl from Acros Organics (Geel, Belgium). Water was of Millipore quality. All other chemicals were of analytical grade.

Preparation of doxorubicin-aconityl-lysozyme conjugate

Doxorubicin was coupled to lysozyme via an acid-labile spacer (fig. 1) using a modified protocol of Shen and Ryser [19]. Of a 2 mg/ml doxorubicin.HCl injection solution, 20 ml was placed on ice in the dark. $Na_2HPO_4.2H_2O$ (89 mg) and *cis*-aconitic anhydride (40 mg) were added while the solution was carefully maintained at pH 9.0 by adding NaOH. After the pH was stable, the solution was stirred for another 30 min. The solution was diluted with 20 ml water and the temperature was raised to approximately 4 °C. EDCI (141 mg) was dissolved in 5 ml phosphate buffer pH 8.0 and immediately added, followed by instant acidification of the solution using HCL. The pH was carefully kept at 5.0 for 10 min. Subsequently, the pH was raised slowly to pH 8.0 by adding NaOH. Lysozyme (42 mg) was then added and the solution was stirred for 20 hours at 4 °C. Non-protein bound compounds were removed in an Amicon stirred cell concentrator using an YM3 membrane (Amicon, Beverly, MA). The product was further purified by gel-filtration (G75, 50 mM phosphate pH 8.5) and again washed with water and concentrated in a stirred cell concentrator. The final solution of conjugate was stored at 4 °C and used within a week for in-vivo studies or lyophilized and stored at - 20 °C.

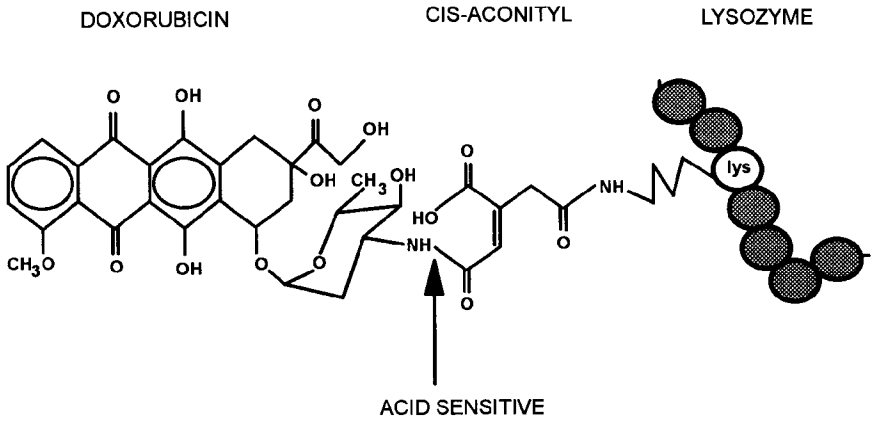

Figure 1. *Chemical structure of doxorubicin-aconityl-lysozyme conjugate*

Characterization of doxorubicin-aconityl-lysozyme

The coupling degree of doxorubicin to lysozyme in the conjugate was determined by measuring the amount of doxorubicin by HPLC and the amount of lysozyme by the method of Lowry [23]. The synthesis was performed using a molar ratio of 25:87:1 doxorubicin:aconityl:lysozyme. This resulted in a conjugate with an average coupling degree of 6.3 mol doxorubicin to 1 mol of lysozyme. Since lysozyme contains 7 free aminogroups allowing drug attachment, an almost complete derivatization of the free aminogroups on the protein was obtained.

The acid sensitivity of the bond between doxorubicin and the *cis*-aconityl linker was tested by incubating (37 °C) conjugate (1 mg/ml) in water diluted 1/1 with phosphate/citric acid (0.1 M pH 4.5). At regular time intervals, 100 µl of incubate was taken and extracted with 250 µl phosphate/citric acid (0.5 M, pH 7.5) and 1 ml chloroform/isopropanol (4:1). After mixing for 1 min, 400 µl organic solvent was evaporated in a bath of boiling water. After taken the mixture to dryness, the residue was dissolved in water/acetonitrile/perchloric acid for doxorubicin measurement by HPLC as described below. Incubation of the conjugate showed a time-dependent release of doxorubicin with a 45% release in 6 hours at pH 4.5, 37 °C. The conjugate contained less than 4% unbound doxorubicin as indicated by the amount of free doxorubicin that was detected at the start of incubation.

In-vivo experiments in rats

Male Wistar rats (Harlan, Zeist, The Netherlands) weighing about 250 g were kept on a low salt diet (0.05% NaCl/20 % protein, Hope Farms Inc. Woerden, The Netherlands) for two weeks. Rats received a single bolus injection of unbound doxorubicin (2 mg/kg), conjugated doxorubicin (2 mg/kg) or conjugated doxorubicin (10 mg/kg), intravenously. Each group consisted of 6 rats. In the first 30 hours, each spontaneously voided urine sample was collected separately to allow an accurate analysis of the urinary excretion kinetics of doxorubicin. Once a week, body weight was measured as indication of general toxicity. Furthermore, 24 hour urine samples were collected once a week to measure the development of proteinuria. After 16 weeks, organ histology was performed in the rats which survived the full 16 weeks of the study (free doxorubicin n = 4, 2 mg/kg conjugated doxorubicin n = 6, 10 mg/kg conjugated doxorubicin n = 5). The rats were anesthetized (Fluothane/O_2). Thereafter, kidneys, liver, heart and lungs were perfused with PBS and processed for histology. Lung tissue was taken to study histological abnormalities of the micro-vasculature.

Histology

Kidney, heart, liver and lung tissues were fixed in neutral-buffered formalin, embedded in paraffin and four micrometer sections were studied using routine histochemical stainings, including H&E, PAS, methenamine-silver and trichrome. The presence and severity of glomerulosclerosis was estimated using a semiquantitative method as described by Raij [24]. Briefly, if 25% of the glomerulus was affected, a toxicity score of 1 was adjudged, 50% was scored as 2, 75% as 3 and a completely damaged glomerulus as 4. The final score is then obtained by multiplying the percentage of glomeruli with the same degree of injury and additions of these scores. A total number of 50 glomeruli per animal was scored moving from cortex to medulla and visa versa. Other histopathological features were studied according to standard procedures.

Analyses

Doxorubicin was determined by a modified HPLC method as described by Hoes [25]. In short, to measure the amount of unbound doxorubicin in urine, the samples were diluted 1/1 with water/acetonitrile and were subjected to HPLC analysis. The total amount of doxorubicin was determined after hydrolysis of doxorubicin to its aglycon. The samples were diluted with HCL to a 1N HCL concentration and incubated for 20 min at 80 °C. The HPLC determination was performed using a Waters 600 controller/pump (Waters,

Milford, MA) and a Waters 717 plus autosampler. The separation column was a μBondapak C18 (30 cm times 3.9 mm I.D.; Millipore Waters, Milford, MA, USA) guarded with a Nova-pak C18 precolumn (Waters). The mobile-phase consisted of water/acetonitrile 1/1 and perchloric acid pH 3.5 for the detection of unbound doxorubicin and doxorubicin-aconityl while water/acetonitrile 1/1 and trichloric acid pH 3.5 was used for analyzing the aglycon of doxorubicin. The flow rate was 1 ml/min. Detection was performed using a fluorescence Jasco 820 EP detector (Tokyo, Japan), operating at excitation wavelength of 485 nm and emission wavelength of 590 nm. HPLC peak integration was performed using the Hewlett Packerd integration system (HP 3395).
Urinary protein concentration was measured by the Biuret method.
All values are expressed as mean ± SEM. To allow presentation of averaged data, the individual voiding times of the rats were synchronized by interpolation of the individual excretion curves. Comparisons were made using Student's t-test.

Results

Urinary kinetics of doxorubicin
Figure 2 shows that after a single injection of 2 mg/kg free doxorubicin in the rat, only 4.4 ± 0.4 % of the drug dose was recovered in the urine in the first 24 hours after administration. In contrast, conjugation to lysozyme resulted in a much higher urinary excretion of doxorubicin: in the 24 hour period 27 ± 1% was excreted in the urine after injection of 2 mg/kg conjugated doxorubicin and 39 ± 3% after injection of 10 mg/kg conjugated doxorubicin. In the present study, we only wanted to study the shift in elimination route of doxorubicin. Therefore, the pH of the urine ranged between 6 and 7 which was unfavorable for uncoupling of doxorubicin from the conjugate. After administration of conjugated doxorubicin, less than 10% of the doxorubicin was recovered in the urine in the unbound form, indicating that after filtration in the glomerulus, indeed the intact conjugate was predominantly excreted in the urine.

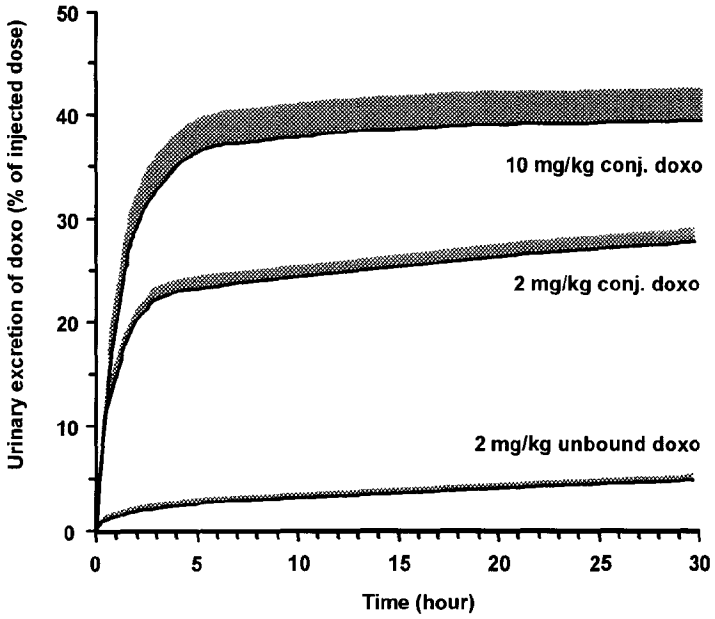

Figure 2. *Cumulative urinary excretion of doxorubicin* (doxo) after intravenous injection of 2 mg/kg unbound doxorubicin (n = 6) and after injection of 2 mg/kg (n = 6) and 10 mg/kg (n = 6) lysozyme-conjugated doxorubicin (conj. doxo). Data are expressed as mean percentage of injected dose. Shaded area indicates the + SEM.

Toxicity of doxorubicin

In rats, especially the kidneys are sensitive to toxic effects of doxorubicin. In these species, doxorubicin induces a nephrotic syndrome which is characterized by proteinuria and glomerulosclerosis. In the present study, after a single injection of 2 mg/kg unbound doxorubicin, all rats developed a marked proteinuria with a maximum of 423 ± 64 mg protein/day after 8 weeks. None of the rats injected with 2 mg/kg or 10 mg/kg conjugated doxorubicin developed proteinuria indicated by a protein excretion in the normal range (14 ± 3 mg protein/day) (fig. 3).

After 16 weeks the rats were terminated for pathology. In all 4 surviving rats that were treated with free doxorubicin, glomerulosclerosis was evident and scored as 65 ± 18. In contrast, none of the rats treated with conjugated doxorubicin showed any signs of glomerulosclerosis. In none of the groups, tubulointerstitial abnormalities, such as interstitial fibrosis or inflammation, tubular dilatation or atrophy nor vascular changes were observed.

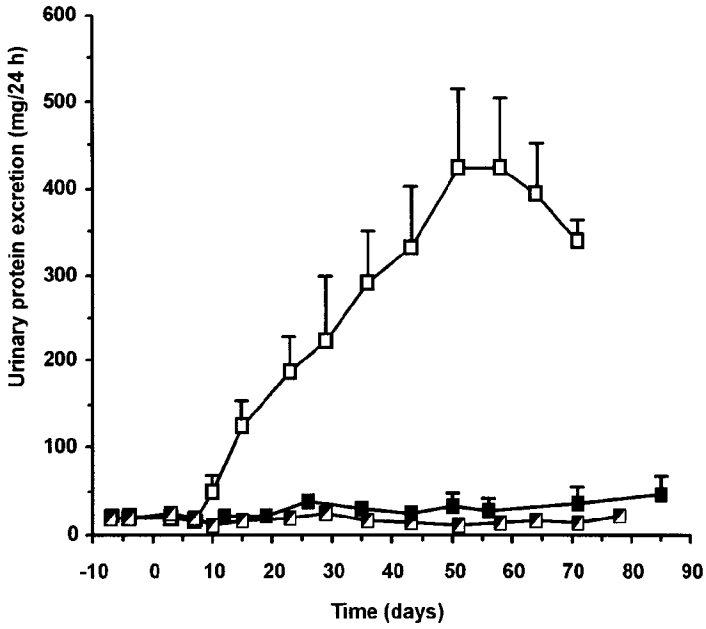

Figure 3. *Time course of urinary protein excretion after intravenous injection.* Open squares: 2 mg/kg unbound doxorubicin (n = 5), black/white squares: 2 mg/kg doxorubicin-aconityl-lysozyme (n = 6), closed squares: 10 mg/kg doxorubicin-aconityl-lysozyme (n = 5). Data are expressed as mean + SEM.

With respect to the general condition of the rat, body weight increased only by 9.8 g/week in the free doxorubicin group whereas this was 13.4 g/week in the two doxorubicin-lysozyme conjugate groups, being significantly different ($p < 0.01$). No signs of histological abnormalities in the cardiac tissue was found in any group of rats. No signs of cardiac atrophy and/or interstitial fibrosis or inflammation were noticed. Inspection of liver and lung tissue neither revealed histological changes.

Discussion

A strategy of targeting to the distal urinary tract of doxorubicin was designed to improve the therapeutic index of the drug in the treatment of bladder carcinoma as visualized in figure 4. The present data show that conjugation of doxorubicin to the LMWP lysozyme leads to an increased urinary excretion of

the antitumor drug without the renal toxic damage as it occurs after administration of the parent drug.

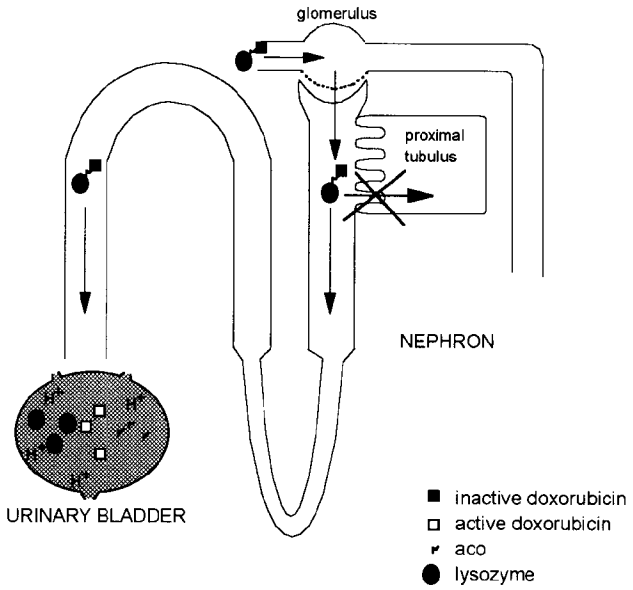

Figure 4. *Schematic presentation of the concept of doxorubicin targeting to the urinary bladder using the LMWP lysozyme as carrier.* Native lysozyme is glomerular filtered and reabsorbed by the proximal tubular cell. By shielding the free amino-groups of lysozyme with doxorubicin-aconityl molecules, the tubular reabsorption will be prevented and the doxorubicin-aconityl-lysozyme conjugate will be excreted into the distal urinary tract. Lowering the pH of the urine will then cause a regeneration of the active drug in the urinary bladder.

Systematically administered doxorubicin is rapidly cleared from the plasma [26] and distributed to various tissues in the body with extensive metabolism [20,21,26,27]. The excretion of the drug and metabolites occurs predominantly via the bile [20,26-29]. In agreement with other studies [20,28], only 4 % of the injected doxorubicin was recovered in the urine in the first 24 hours after injection. Conjugation of doxorubicin to the LMWP lysozyme however, resulted in an 7 to 9 fold increase in the cumulative urinary excretion of doxorubicin.

Doxorubicin (or adriamycin) is a toxic drug that can damage many different organs and tissues, including kidneys, heart, blood vessels and bone marrow. However, there appears to be species difference in the relative severity of toxicity to the various tissues. In humans, doxorubicin is mainly cardiotoxic [22] whereas in the rat, especially the kidneys are sensitive for the detrimental effects of this antineoplastic agent [22,30]. As the consequence of renal toxicity of doxorubicin, the rat develops a nephrotic syndrome, characterized by massive proteinuria and glomerulosclerosis [22,30-34]. In our hands, injection of 2 mg/kg doxorubicin induces a nephrosis with well defined characteristics [34]. In agreement with this, the 2 mg/kg dose of free doxorubicin in the present study induced a marked and relatively stable proteinuria and glomerulosclerosis. In contrast, when 2 mg/kg doxorubicin was administered in the conjugated form, i.e. doxorubicin was coupled to the LMWP lysozyme, no proteinuria and glomerulosclerosis was observed. Even the extremely high dose of 10 mg/kg conjugated doxorubicin did not induce any detectable damage to the kidneys. This is the more striking realizing that 45 times more doxorubicin was cleared by the kidney after administration of 10 mg/kg conjugated doxorubicin than after dosing 2 mg/kg unbound drug. With respect to other organs, doxorubicin itself as well as its lysozyme-conjugate did not induce detectable abnormalities elsewhere in the body even at the high dose of conjugated doxorubicin. The increase in body weight of the rats treated with parent drug was slower than that of the rats treated with doxorubicin-lysozyme conjugate, indicating that conjugated doxorubicin was better tolerated than the free drug.

Although we observed a clear shift in the excretion route of doxorubicin in favor of the urine in case it was conjugated to lysozyme, only 27% to 39% of the injected dose was recovered in the urine during the first day after injection. One possible explanation for this incomplete urinary excretion could be that the tubular reabsorption of lysozyme was not fully prevented by derivatization of its free amino-groups [17]. Secondly, the increase in hydrophobicity of the lysozyme molecule due to coupling of 5 to 7 doxorubicin groups may stimulate tubular endocytosis of the conjugate in spite of the shielded aminogroups. However, if this would be the case, one should expect at least some tubular damage. An isolated tubulo-interstitial toxicity was not found though.
The incomplete urinary recovery of conjugated doxorubicin could also be explained by extra-renal clearance of the conjugates. Yet, no histological abnormalities were found in the lung, heart and liver tissue. At the given doses, these tissues may be protected from doxorubicin toxicity by sufficient

antioxidant defense mechanisms [35]. Thus, a partial removal of conjugate by the reticulo-endothelial system may occur but under the present experimental conditions seems not a serious drawback. However, more detailed studies on the cardiac but especially the hepatic distribution and toxicity of the conjugate are required [36].
Before clinical development of the proposed strategy, the conjugate should be tested for effective release and action of the drug in the urinary tract. In-vitro, the conjugate was stable at pH 7.4 with a time-dependent release of free doxorubicin at more acid conditions. Shen and Ryser [19] showed that an intact conjugate of doxorubicin-aconityl-antibody was not cytotoxic. In contrast, after acid hydrolysis the fully active drug was generated. For treating bladder carcinoma in patients, acidification of the urine may be achieved by oral administration of ammoniumchloride or ascorbinic acid [37] combined with an administration of the conjugate at the beginning of the night. The doxorubicin conjugate present in the urinary bladder during the night could then be hydrolyzed and an adequate exposure of the bladder tumor to the regenerated doxorubicin could be produced. At pH 5-6 we expect that at least a part of the conjugate will be split during residence of the conjugate for 6 to 8 hours in the bladder. To further improve the proposed drug targeting strategy, the use of a more optimal pH-sensitive spacer with a higher rate of hydrolysis in a higher pH range might also be considered [38]. Alternatively, a drug-protein linkage may be used that is cleaved enzymatically in the tubular lumen of the kidney [39,40]. This way, doxorubicin may even be effective in the treatment of upper urinary tract tumors, tumors which are highly resistant to treatment. Finally, other antineoplastic agents, such as mitomycin with an optimum antitumor activity at low pH [41] as well as (polymeric) drug carriers [42] could be considered to improve the proposed strategy.

The clinical relevance of bladder targeting of doxorubicin remains to be demonstrated. At the moment, patients with a superficial bladder carcinoma are treated weekly through an intravesical irrigation with a solution of the antitumor drug [43]. The particular treatment is inconvenient for the patient while local irrigation often leads to cystitis [5,44]. Besides, intravesical irrigation is a time-consuming and laborious procedure compared to other methods of administration. Of note, very little is known about the intravesical irrigation procedure with respect to optimal drug concentration, pH conditions and duration of the irrigation in relation to the effect of the treatment [5,45]. As an alternative, drug-protein conjugates as described in the present study can be administered intravenously, or maybe even subcutaneously. This general route

of administration may be more convenient and still would allow manipulation of the dosage regime in order to attain optimal therapeutic conditions for anticancer therapy. Among others, through a standardized diet and addition ingestion of acidifying agents an optimal pH range of bladder urine could be established in relation to the hydrolysis profile of the chosen conjugate.
In conclusion, an increased urinary excretion and reduced toxicity of doxorubicin was obtained by coupling 5 to 7 doxorubicin molecules to the LMWP lysozyme via an acid-labile linker. This procedure may improve the exposure of the antitumor drugs to the urinary bladder. At present, studies are initiated to test the efficacy of the conjugate in experimental bladder tumors in the rat.

Acknowledgment

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Chapter 8

Urine collection in the freely moving rat: reliability for measurement of short-term renal effects

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Abstract

Studies on short-term renal responses to (pharmacological) intervention require accurate and multiple collection of urine samples. Several invasive techniques have been described for frequent urine collection of the conscious rat, each having their own limitations. No data are available about the feasibility of the spontaneously voiding, freely moving rat for this purpose. In the present study, bladder voidings of six rats were time-registered and collected separately for several days. The data show a considerable 24 hour variation coefficient of both the voided volume and of the bladder collection time with a poor correlation between the two parameters. Forced diuresis induced by continuous i.v. infusion (2 ml/h) increased the frequency of urine voiding and thus the time-resolution of the urine-production pattern. However, this method failed to reduce the variation coefficient of the voided volume, the collection time and the correlation between the two parameters. The fact that variations in creatinine excretion paralleled the variation in urinary flow suggests that both phenomena are likely be due to incomplete bladder emptying. Correction for this incomplete bladder collection, using the creatinine excretion, indeed reduced the variation coefficient of sodium excretion successfully from 61 ± 17% to 29 ± 5% during normal diuresis and from 56 ± 19% to 22 ± 6% during forced diuresis.

In conclusion, the spontaneously voiding, freely moving rat can be used for short-term renal response studies if the collected urine samples are corrected for incomplete bladder emptying using urinary creatinine concentrations. This procedure allows the detection of changes in a urinary parameter if this exceeds a 40% deviation of the normal value.

Introduction

The conscious rat is an attractive model to study renal (patho)physiology and related pharmacological interventions. In contrast to the anesthetized rat, there is no potential interference by anesthesia [1] and fewer limitations in the duration of the study. The individual rat may thus serve as its own control if subjected to different pharmacological interventions. However, the conscious animal model may also have its disadvantages. For short-term renal studies, accurate timed urine collection is essential. Under anesthesia, this can be rather easily obtained by cannulation of the bladder or even of the urethras. In order to obtain an accurate urine collection in the conscious rat things are far more complicated. Several approaches have been taken such as forced micturition, and collection through acute or chronic catheters with various implanting devices [2-9]. While stress is the major drawback of using forced diuresis and acute implantations, infection and obstruction are the risk factors of the chronic invasive devices. Since the "true" physiological state of the rat is most likely best represented by the conscious, unrestrained rat that micturates at free will, this approach is to be preferred. However, no data are available on its feasibility to study short-term renal effects under these experimental conditions. Therefore, in the present study the micturition pattern of the spontaneously voiding, freely moving rat was tested. The accuracy and time-resolution of the model was determined during normal and forced diuresis and the impact on the renal parameters to be determined was established.

Methods

Materials

Polyphenyl pyrrolidone 25 (PVP) was purchased from Serva (Heidelberg, Germany). Creatinine and dopamine.HCl were obtained from Sigma Chemicals Co. All chemicals used for analytical purpose were of analytical grade. Water was of Millipore quality. Infusion solutions were sterile.

Urine collection system

A plastic metabolic cage with a wire mesh floor (Tecniplast Gazzada, Buguggiate, Italy) was placed above a fraction collector (model 2110, Bio-Rad Laboratories, Inc). The times of voiding were recorded and the individual voidings collected separately using computer communication . The computer

connections were made via an I/O interface. The software was written in Borland C/C^{++}.

Experimental set-up

Six male Wistar rats (Harlan, Zeist, The Netherlands) weighing 250 gram were kept in a temperature controlled room with a 12/12 h light/dark cycle. They were given solid chow (Hope Farms Inc., Woerden, The Netherlands) and tap water ad libitum throughout the study.

Study 1 (normal diuresis): Six rats were transferred to individual metabolic cages. After a stabilization period of 14 hours, the spontaneously voided urine samples were time recorded and collected separately for five days.

Three days after the first study, the rats were anesthetized with 2% Forene/O_2 (500 ml/min) and the jugular vein cannulated (Silastic, medical grade tubing (.020 in. ID x 0.037 in. OD) from Dow Corning (Midland, Michigan, U.S.A)). The cannula was subcutaneously pulled under the skin to the head and immobilized with screws and dentist-cement (Simplex (Rapid), Associated Dental Products, Kemdent Works, Purton, Swindon, England). To protect the cannula from blood clotting, the cannula was filled with 50% PVP/500 IE/ml heparin in 0.9% NaCl when not in use [10]. The rats were allowed to recover from surgery for one week, after which the second study was performed.

Study 2 (forced diuresis): Six rats were transferred to their metabolic cages and constantly infused with 0.45% NaCl/2.5% glucose (2 ml/h). Again, after a stabilization period of 14 hours, urine samples were collected for five days.

Study 3 (dopamine): A rat was challenged with dopamine. After a stabilization period of 18 hours, 20 µg/min/kg dopamine was infused for one hour through the permanent jugular cannula. Urine samples were collected starting five hours before and finishing ten hours after the start of the dopamine infusion.

Analytical methods

The urine volume was measured by weighing the collected samples (accuracy of 0.02 gram). Urinary creatinine was measured by HPLC as described by Nishimaki et al. [11]. In short, the mobile phase consisted of 96.5% 20 mM KH_2PO_4/K_2HPO_4, pH 5.1 containing 80 mg/l sodium lauryl sulphate, and 3.5% acetonitrile. The separation column was a µBondapak C18 (30 cm x 3.9 mm I.D.; Millipore Waters, Milford, MA) guarded with a µBondapak C18 Guard-pak precolumn (Millipore Waters). The injection volume was 20 µl (Jasco autosampler, model 851-AS, Tokyo, Japan), the flow rate 1 ml/min (Jasco pump model 880 PU) and the detection wavelength 230 nm (Jasco UV-detector, model 875-UV). HPLC peak integration was performed using the integration

package JCL6000 (Jones Chromatography, Littleton, CO). Urine samples were diluted 50 to 100 times in water and injected on the column without a pre-purification. Pure creatinine dissolved in water was used as standard. Urine sodium concentrations were measured by flame photometry (Perkin Elmer 3030B Atomic Absorption Spectrophotometer).

Creatinine correction

Na excretion (µmol/min) *corrected* = Na excretion (µmol/min) / creatinine excretion (µmol/min) x average creatinine excretion in 24 hours (µmol/min).

Results

Micturition characteristics

The individual voided urine volumes and corresponding bladder collection times (the time between two bladder emptyings) of six rats on day 4 under normal diuresis (*study I*) are given in figure 1A. The variation coefficient of both the voided volume and collection time was large (table 1) and the correlation between the two parameters appeared to be poor (day 4; r = 0.69, n = 83). The variation in voided volume did not change in the course of the study as indicated by an average individual variation coefficient of 61, 62, 56, 60 and 50% on day 1, 2, 3, 4 and 5, respectively. Similarly, the bladder collection time varied to the same degree over the 5 days of the study with an average individual variation coefficient of 80, 61, 70, 61 and 58%, respectively.

Tabel 1: Micturition characteristics and renal parameters of the spontaneously voiding rat.

Diuresis:	**Normal**		**Forced**	
n = 6, day 4	mean	VC (mean ± SD)%	mean	VC (mean ± SD)%
voided volume (ml)	0.6	60 ± 23	1.6	47 ± 18
collection time (min)	96	61 ± 14	60	66 ± 20
U-flow (µl/min)	7.3	51 ± 11	34	64 ± 22
Creat. excretion (µmol/min)	0.10	55 ± 13	0.09	53 ± 22
U-flow (µl/min) *corrected*	6.4	21 ± 10	27	31 ± 7
Na excretion (µmol/min)	2.2	61 ± 17	5.7	56 ± 19
Na excretion (µmol/min) *corrected*	1.9	29 ± 5	4.6	22 ± 6

Urinary flow (U-flow) and sodium excretion (Na excretion) *corrected* indicate a correction for creatinine excretion: renal parameter/creat excretion x average creat excretion in 24 hours.
VC = variation coefficient.

A forced diuresis (*study 2*) was induced with the intention of increasing the time-resolution of the method and to increase the accuracy of urine collection. Forced diuresis resulted in a 2.7 fold increase of the voided volume and 1.6 fold reduction of collection time (table 1). This implied a ± 5-fold increase of the urinary flow. Yet, the variation coefficient of voided volume and collection time remained high and the accuracy of urine collection did not improve but rather was reduced by forced diuresis as indicated by a lower correlation between voided volume and collection time (r = 0.33, n = 141, figure 1B).

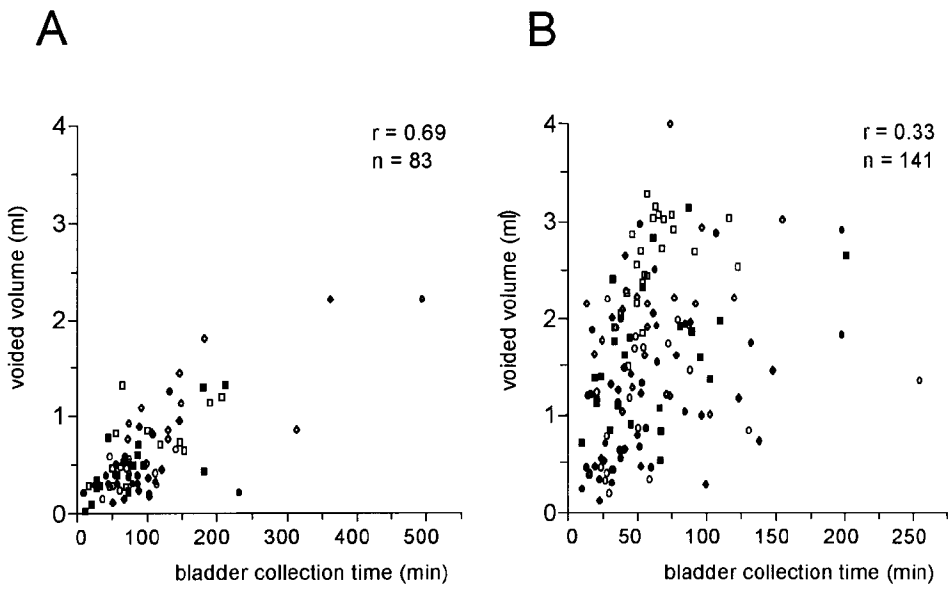

Figure 1. *Correlation between the individual voided volumes and collection times of six rats on day 4 of the study.* A: during normal diuresis. B: during forced diuresis. The six symbols represent the six rats.

Renal excretion parameters

As expected from the micturition data, the urinary flow of individual urine voidings fluctuated considerably in time, from less than 1 to more than 20 µl/min. It is interesting to note that the creatinine excretion, which is supposed to be rather constant over the day, showed a fluctuation parallel with the variation in urinary flow. A high correlation was found between the urinary flow and creatinine excretion of individual bladder voidings at day 4 both under

normal diuresis ($r = 0.92$, $n = 83$) and forced diuresis ($r = 0.81$, $n = 141$), both $p < 0.0005$ (figure 2). These data suggest that the observed variations are explained by incomplete bladder emptying of the rat. Correction for creatinine excretion clearly smoothed the 24 hour sodium excretion pattern of an individual rat (figure 3) with an improvement of the variation coefficient of urinary flow and urinary sodium excretion by a factor 2 to 3 (table 1).

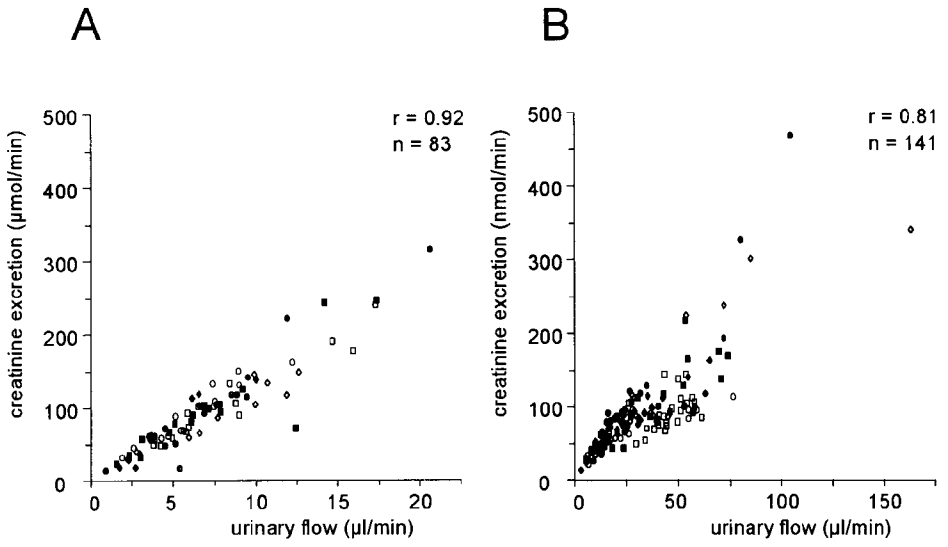

Figure 2. *Correlation between the urinary flow and creatinine excretion of the individual voidings of six rats on day 4 of the study.* **A: during normal diuresis. B: during forced diuresis. The six symbols represent the six rats.**

In the typical example (see *study 3,* figure 4), dopamine was infused for one hour starting directly after the bladder emptying of urine voiding no. 6. The large, short lasting peaks of natriuresis no. 6 and 11 in the uncorrected graph (figure 4A) were removed using creatinine correction (figure 4B). This indicates that those peaks were likely due to incomplete bladder voiding. As a result, after creatinine correction, the natriuresis induced by dopamine infusion was more clearly visualized.

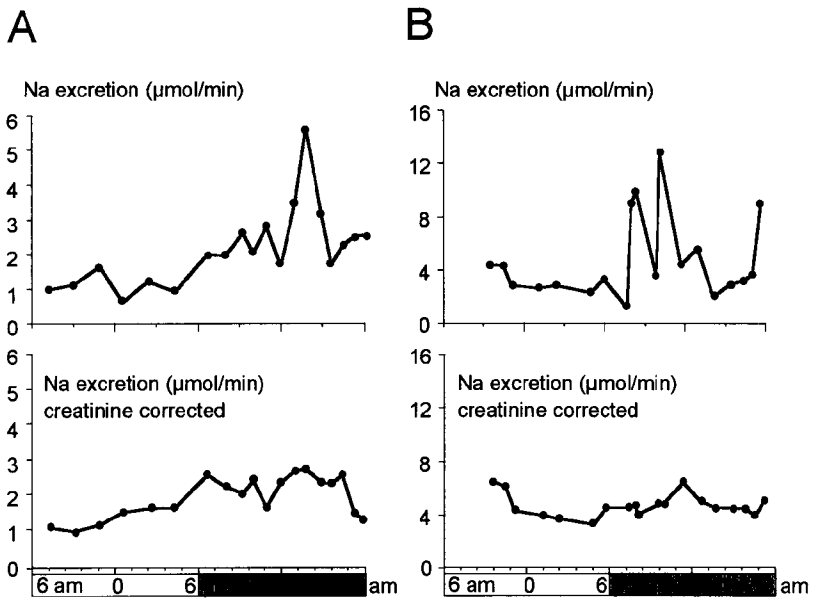

Figure 3. *A typical example of a 24-hour profile of sodium excretion of an individual rat.* On top, the sodium excretion without and on the bottom, the sodium excretion with creatinine correction. A: during normal diuresis. B: during forced diuresis.

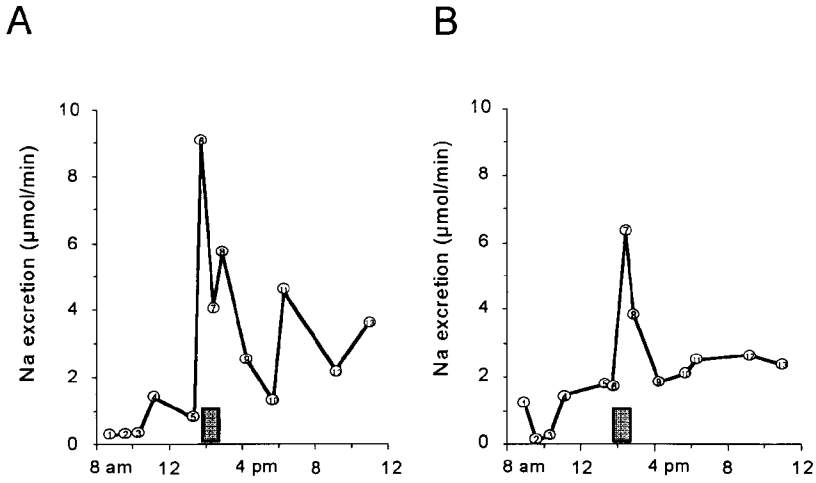

Figure 4. *A typical example of the sodium excretion pattern of an individual rat challenged with dopamine.* ▯ : one hour i.v dopamine infusion (20 µg/kg/min). A: no corrections, B: with creatinine correction.

Discussion

The spontaneously voiding, freely moving rat is likely to be a better representative of the "true" physiological state of the animal compared to rats under anesthesia or rats undergoing invasive implantation procedures. However, the present study shows that the feasibility of using this model for short-term renal studies is rather limited due to incomplete bladder voiding. During normal diuresis, the voided volume and collection time appeared to be highly variable and poorly correlated. Territorial behavior or adaptation to the metabolic cage is unlikely to be responsible for this large fluctuation: in the five days of the study, the housing part of the metabolic cage was not cleaned and the rat was not removed from the cage. Evidently, if territorial behavior or adaptation were a major factor we would have expected the variation in the micturition parameters to decrease in time, which was not the case. Furthermore, female rats, often with less territorial behavior, showed a similar degree of variation in voided volume as their male littermates [12].

We induced a forced diuresis with the intention of increasing the bladder filling equally over the 24 hours cycle, this to improve the accuracy of urine collection. Unexpectedly, the induced increase in urinary flow reduced rather than improved the accuracy of urine collection. With regard to the time-resolution, a 5-fold increase in urinary flow resulted in only a 1.6 fold increase in time-resolution since both the frequency and volume of the bladder voidings were affected. Apparently, the bladder can readily adapt to changes in diuresis by adjustment of capacity without changing the completeness of bladder emptying during voiding.

In contrast with the forced diuresis protocol, creatinine correction was successful in increasing the accuracy of urinary parameters. Creatinine excretion can be used for correction of incomplete bladder emptying since creatinine is constantly produced throughout the body's musculature and subsequently excreted via the kidney [7,9].

To test the value of the method of creatinine correction in the spontaneously voiding rat model for the detection of short-term changes in renal excretion parameters, the stability of sodium excretion over the day was also measured. Without correction for incomplete bladder voiding, the model seemed not very sensitive. With a coefficient of variation of about 60%, the method only allowed an accurate measurement when at least a 2-fold change in sodium excretion was present. Creatinine correction however resulted in a marked reduction of the coefficient of variation allowing the measurement of a ± 40% change in sodium excretion.

In conclusion, creatinine correction makes the spontaneously voiding, freely moving rat an attractive model for short-term renal intervention studies. Without this correction for incomplete bladder voiding only large changes in renal excretion parameters are detectable. If the creatinine correction procedure is used the model is also applicable for detection of less pronounced pharmacological effects.

Acknowledgment

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Summary and general discussion

Overseeing the present state of the art of drug targeting to the kidney one can say that there are many opportunities. Several approaches have been explored, focusing on the delivery of several kinds of drugs to several sites within the nephron. Yet the procedures proposed so far are still in the preclinical exploration phase. The largest progress was probably made in the development of renal prodrugs. This is not surprising taken into account the versatile experience that could be adopted from bioavailability and brain delivery research. Although the design and development of prodrugs is appealing, there are still several limitations to these formulations with regard to cell-specificity [Chapter 1]. We propose selective drug targeting to the kidney as well as to the urinary bladder using low-molecular-weight proteins (LMWPs).

Renal handling of low-molecular-weight proteins

Are LMWPs specifically taken up and catabolized in the kidney?
Comparison of the kinetic features of different LMWPs revealed that all tested LMWPs are rapidly cleared from the general circulation with a rapid accumulation in the kidney [Chapter 2]. Besides the kidney, the LMWPs appeared not to accumulate elsewhere in the body. After renal uptake, the LMWPs were catabolized with distinct individual differences in catabolic rate [Chapter 3]. From other studies we know that the renal accumulation of LMWPs is a result of free filtration with a subsequent reabsorption in the proximal tubular cells. The catabolism of the LMWPs takes place in the intracellular lysosomes of the proximal tubular cells [1,2]. As such, LMWPs are suitable to serve as renal specific drug carrier: the drug-LMWP conjugate will be rapidly removed from the circulation and taken up by the kidney while unwanted sites of action elsewhere in the body will be avoided using this method. The delivery into lysosomes allows drug-carrier attachment via several kinds of bonds. The lysosomes are stacked with a variety of proteolytic enzymes in an acidic environment. Drug release from a drug-carrier conjugate may therefore be achieved using peptide, ester or acid-labile bonds between the drug and protein carrier. The differences in rate of catabolism between LMWPs as well as the rate of hydrolysis of the bond between the drug and carrier may be used to manipulate the rate of drug release in the kidney. Also, the migration time of the endocytosed conjugate from the luminal membrane to the proteolytic lysosomes could be a rate limiting step in renal LMWP

catabolism. Consequently, the LMWP concept of drug targeting allows a controlled drug release that can be manipulated at various stages of the renal disposition process. For instance, for chronic treatment, a slowly processed LMWP might be preferred as a drug carrier while for a pulse treatment a rapidly processed protein (with a short migration time and/or an endosomal release mechanism) could be a more appropriate choice.

Does proteinuria affect the renal handling of LMWPs?

Proteinuria is one of the most prominent abnormalities found in renal diseases and is partly responsible for the progression of renal disease [3]. As a consequence of the glomerular leakage of proteins, the proximal tubular cells are exposed to an increased amount of protein. This may seriously affect renal drug targeting using LMWPs. One may expect a drug-LMWP conjugate to compete with the overload of protein for tubular uptake as well as for catabolism. We showed that the amount of renal uptake of the LMWPs was minimally affected by proteinuria [Chapter 4]. In contrast, the rate of LMWP catabolism was reduced by proteinuria. For drug targeting strategies this indicates that drug delivery to the proximal tubular cell will not be hindered by proteinuria. However, depending on the mechanism underlying the reduced catabolism as well as depending on the chosen drug release mechanism, drug release may or may not be affected by proteinuria. Saturation of the proteolytic enzymes in the lysosomes may reduce the rate of drug release if enzymatic cleavage is required. In contrast, targeting with drugs that are attached with an acid-labile spacer may in that case not be affected by proteinuria. This study also showed that the effect of proteinuria on LMWP catabolism was dependent on the proximal tubular segment of LMWP handling. Presumably, the overload of filtered albumin during glomerular leakage is preferentially taken up by the late proximal convoluted tubular cells since especially in that segment, LMWP catabolism was affected. These data also allow us to speculate that, through coupling to proteins filtered by the glomerulus, drugs can be delivered specifically to those proximal tubular cells that are predominantly affected by proteinuria. This might be essential for drugs chosen to protect the tubular cell from further damage by proteinuria. In addition, it is possible to use certain LMWPs as drug carriers to circumvent the proteinuria-affected cells. In this case, treatment (for example, for proteinuria unrelated diseases) will not be hindered by the severity of proteinuria.

Drug targeting to the kidney

What are the kinetics of naproxen-lysozyme conjugate?

Targeting of non-steroidal anti-inflammatory drugs such as naproxen to the kidney could be of interest because they are beneficial in the kidney but toxic elsewhere in the body [4,5]. Naproxen was coupled to the LMWP lysozyme through an amide bond between the carboxylic group of naproxen and available free amino-groups of the protein.

First, a study was performed to examine the kinetic features of the conjugate [Chapter 5]. Conjugation with naproxen did not affect the characteristics of the LMWP lysozyme. Like native lysozyme, the conjugate rapidly accumulated in the kidney with a subsequent slow intrarenal degradation. Thus, in spite of conjugation, lysozyme remained renal specific and accessible to degradation. Focusing on the drug moiety of the conjugate we found that conjugation of naproxen to lysozyme distinctly altered the kinetics of the drug. Conjugation to lysozyme resulted in a pronounced increase of naproxen accumulation in the kidney. Up to now, no renal targeting strategy has shown such a relatively high enrichment of a drug in the kidney [Chapter 1]. After delivery to the kidney, naproxen was gradually released from the conjugate as indicated by the reduction of renal amount of naproxen in time. An important observation with regard to the aimed reduction in extra-renal side-effects was that no detectable amounts of free naproxen were present in the plasma at any time after administration of conjugate.

Does the naproxen-lysozyme conjugate show renal effects?

Having obtained these promising kinetic data, we investigated whether naproxen-lysozyme exhibits renal effects. Naproxen, as an inhibitor of cyclo-oxygenase, blocks prostaglandin synthesis. Among others, naproxen reduces furosemide-stimulated urinary excretion of prostaglandin E_2 (PGE_2) as well as the natriuretic and diuretic effects of furosemide. Our study demonstrated that naproxen-lysozyme treatment prevents furosemide-induced excretion of PGE_2 indicating the renal generation of active compounds from the conjugate. Surprisingly, the effect occurred in the absence of a change in natriuretic and diuretic response to furosemide. In this respect the pharmacological effect differed from treatment with a high dose of free naproxen. We can only speculate about the difference in effect between conjugated and free naproxen. One possibility is a difference in intrarenal kinetics of the NSAID compounds involved. Free naproxen is extensively reabsorbed in the distal tubule of the kidney [6] via which route it may effectively inhibit prostaglandin synthesis in

the medullary interstitial cells. On the other hand, naproxen-lysine, the active metabolite that is released from the naproxen-lysozyme conjugate, is more hydrophilic and may be unable to reach these sites of prostaglandin synthesis involved in the furosemide-induced excretion of sodium and water [7].

Drug targeting to the urinary bladder

Can doxorubicin be targeted to the urinary bladder by conjugation to lysozyme?

Drug targeting to the urinary bladder could be of interest for antitumor drugs such as doxorubicin that are effective in the treatment of bladder carcinoma but have a low urinary excretion and are highly toxic elsewhere in the body [Chapter 7]. We studied the targeting of the antitumor drug doxorubicin that was coupled via an acid-sensitive spacer to the LMWP lysozyme. To prevent tubular reabsorption of the conjugate, almost all positively charged groups of lysozyme were shielded by coupling to doxorubicin molecules. An acid-sensitive spacer was intercalated between doxorubicin molecules and lysozyme to allow drug release after arrival in the urinary bladder, following urine acidification. As expected, conjugation to lysozyme resulted in a marked increase in the urinary excretion of doxorubicin. Since we investigated the potential shift in elimination route at a neutral pH range of the urine, the major fraction of the doxorubicin excreted in the urine after conjugate administration was still protein bound. In-vitro however, the acid-sensitivity of the bond between doxorubicin and the linker was confirmed. For the treatment of bladder carcinoma in patients, acidification of the urine may be achieved by oral administration of ammoniumchloride or ascorbic acid. This approach is under further study.

Does conjugation to lysozyme prevent renal toxicity of doxorubicin?

Subsequently, we tested if conjugation to lysozyme reduced doxorubicin toxicity. In the rat, especially the kidneys are sensitive to the detrimental effects of this antineoplastic agent [8]. As a consequence of renal toxicity, the rat develops a nephrotic syndrome, characterized by massive proteinuria and glomerulosclerosis. In contrast to free doxorubicin, the lysozyme conjugated form did not induce proteinuria and glomerulosclerosis. Even an extremely high dose of 10 mg/kg conjugated doxorubicin did not have any detectable damaging effect to the kidney.

Conclusion

LMWPs are suitable to serve as specific drug carriers to the kidney. The concept appears applicable in the proteinuric state since renal uptake and catabolism of LMWPs remain during proteinuria. Tubular site and rate of drug release may be manipulated by choosing a proper LMWP carrier on the basis of its site of tubular uptake and its rate of renal catabolism.
The NSAID naproxen can be delivered specifically to the kidney with a subsequent release of renal NSAID activity using the LMWP lysozyme as a carrier. Whether the conjugate is effective in the treatment of certain renal diseases is the objective of future studies.
The urinary excretion of the anti-tumor drug doxorubicin can be increased by a "high degree" coupling to lysozyme. Furthermore, conjugation to the LMWP prevented the renal toxicity of doxorubicin. Whether the drug can be adequately released from the acid-labile conjugate *in-vivo* in acidified urine and whether bladder carcinoma can be treated using this conjugate remain questions to be answered.

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Perspectives

This thesis demonstrates the feasibility to use low-molecular-weight proteins (LMWPs) for delivery of drugs specifically to the kidney and to the urinary bladder with the potential regeneration of the active drug or metabolic products in the proximal tubular cell of the kidney or the urinary space. In the following section several suggestions are offered for future research:

Treatment of chronic renal disease

In this thesis we describe, for the first time, a renal effect of a drug targeted to the kidney using a LMWP carrier. An intravenous injection of the non-steroidal anti-inflammatory drug naproxen coupled to the LMWP lysozyme prevented the physiological response of an increased renal prostaglandin synthesis following furosemide administration. An attractive follow-up of these studies would be to investigate whether naproxen-lysozyme conjugate is effective in treating proteinuria and tubular defects. This should occur without inducing major side-effects as produced by non-targeted naproxen. Continuation of this research will be important to elucidate the clinical potential of the naproxen-lysozyme conjugate in specific diseases and the potential of the drug-LMWP strategy in the treatment of chronic renal diseases in general.
Disadvantages of the LMWP-strategy for the treatment of chronic renal disease are the requirement of parenteral administration and the possible immunogenicity of the drug conjugates. With respect to the administration route, possibly the conjugate can be administered subcutaneously like the LMWP insulin. If immunogenicity would mean a serious limitation for chronic treatment, synthetic polymers may be used as carriers instead.

Treatment of acute renal risks

The LMWP-strategy may be of interest for the treatment of renal risks that require acute therapy, such as protecting the kidney during acute reperfusion and preventing allograft rejection after transplantation [1]. For these short-term clinical interventions, parenteral administration will not be a limitation.

Diagnostics

In collaboration with prof. dr. D.N. Reinhoudt of the Organic Chemistry Department of the University of Twente the LMWP concept was used to improve the rubidium/krypton technique for measuring renal blood flow [2]. The idea is still very relevant for clinical as well as experimental purposes. The research may be continued when the main problems concerning the high lipophilicity of the rubidium complexing agent are solved.

Renal kinetics and dynamics

The interest in renal drug targeting is not necessarily limited to improve therapy. The strategy may also be very helpful in answering (patho)physiological and pharmacological questions. A still unanswered pharmacological question is at what site in the kidney drugs should be delivered to achieve their desired action. The LMWP strategy may help to clarify these questions. Intriguing for example was the observation that the naproxen-lysozyme conjugate did reduce the renal excretion of PGE_2 but did not affect sodium and water excretion, in contrast to a high dose of free naproxen.
Another important research issue for which the drug-LMWP targeting strategy may be an interesting tool to use, is the role of the proximal tubule in progression of renal failure and the therapeutic impact of a selective drug interference in tubular (patho)physiology [3,4].

Drug targeting to the glomerulus

Apart from the proximal tubular cell, other renal sites of drug delivery might be of interest to study. Up to now only a minimal degree of glomerular drug enrichment has been achieved (chapter 1). One suggestion for glomerular drug targeting is the use of liposomes as drug carriers coupled with specific antibody fragments. The glomerular specificity will be achieved by using antibody fragments directed against glomerular targets or targets of inflammation. The drug can be easily incorporated in the liposome without the requirement of troublesome coupling procedures as required for the LMWP strategy. All facilities and expertise needed for the development of such constructs are present in the Groningen Institute of Drug Studies.

Cancer therapy in the urinary tract

The doxorubicin-aconityl-lysozyme study (chapter 7) showed promising results for a clinical applicability of the strategy to treat cancer in the urinary bladder. Follow-up studies are planned to measure the therapeutic effects in an experimental model of bladder cancer and to further optimize this strategy.
Apart from this, we are planning to examine the potentials of LMWP targeting to treat renal cell carcinoma, a cancer that is highly resistant to the convential way of treatment. Recently, a project was started with prof. dr. H.J.A Mensink of the Urology Department and dr. D.A. Piers of the Nuclear Medicine Department to examine the renal uptake pattern of a LMWP in patients with different degrees of renal cell carcinoma. This study may tell us whether LMWPs are taken up by the tubular cancer cells and/or healthy tubular cells surrounding the tumor. Even if the LMWP is not taken up by the tumor cells themselves, conjugation of an antitumor drug to a LMWP could improve renal cancer therapy: compared to free drug, extrarenal toxicity will be reduced while a larger amount of active drug is concentrated close to the tumor cells. Possibly, toxicity for the healthy cells may be circumvented by using a bond that releases the drug already in the tubular lumen. Except for cytostatic agents, the LMWP-strategy may also be applicable for local radiation therapy or as a diagnostic marker for renal cell carcinoma.

Genes and oligonucleotides

Genes are far too large to pass the glomerular basal membrane and can therefore only be targeted to the glomerulus. Interestingly, it has been demonstrated that systemically administered phosphorothioate oligonucleotides accumulate to a substantial amount in the proximal tubular cells of the kidney [5,6]. Thus, a renal specific targeting of oligonucleotides can be obtained but should be further improved by, for example, complexing the nucleotide with poly-lysine to prevent liver uptake.

In conclusion

There are numerous highly relevant goals to achieve in the field of drug targeting to the kidney and to the urinary bladder. Participation by various disciplines will provide essential technologies from basic medicinal chemistry research to physiological effect studies in patients. The research of drug targeting is not only valuable for defined goals. As a result of its applicability and multidisciplinarity, drug targeting research raises (patho)physiological and pharmacological questions that are normally not raised.

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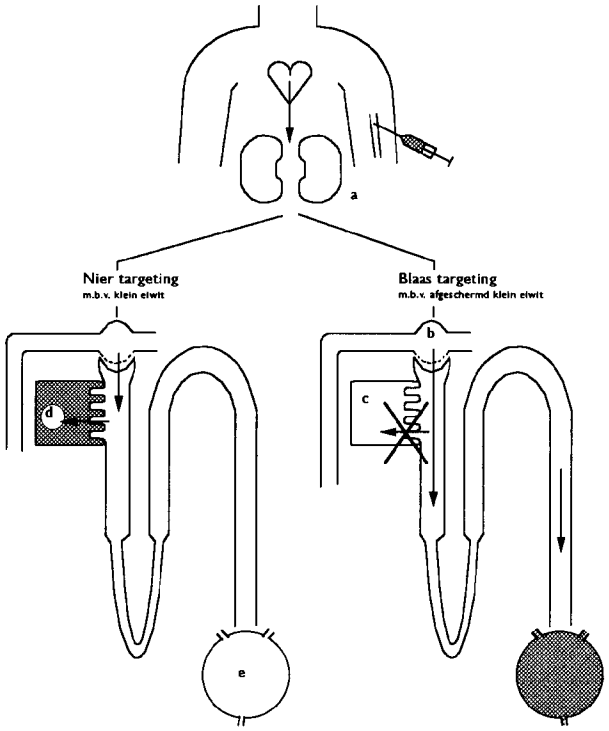
Samenvatting

"Dit geneesmiddel kan duizeligheid, misselijkheid, kriebelhoest, haaruitval, blindheid etc. veroorzaken".
Dergelijke waarschuwingen in een bijsluiter wijzen er op dat het geneesmiddel niet alleen de ziekte behandelt maar ook elders in het lichaam allerlei narigheid veroorzaakt. Het sturen van een geneesmiddel naar de gewenste plaats van werking zodat de bijwerkingen worden voorkomen wordt "drug targeting" genoemd.
In het hier beschreven promotieonderzoek zijn de mogelijkheden onderzocht van "drug targeting" naar de nier en de blaas.

De geneesmiddeldrager

Een belangrijk onderdeel voor het succes van "drug targeting" is de keuze van de stof die het geneesmiddel naar de gewenste plaats in het lichaam brengt.
De nier is opgebouwd uit een groot aantal filter-eenheden, de zogenaamde nefronen. In het filter (de glomerulus) wordt het bloed gefiltreerd. Grote eiwitten en bloedcellen worden door het filter tegengehouden. De overige stoffen gaan door het filter heen en stromen via een buisje (tubulus) naar de blaas. De tubulus is opgebouwd uit gespecialiseerde cellen (tubuluscellen) die belangrijke stoffen uit het filtraat opnemen en er afvalstoffen aan toevoegen. Aan het eind komen alle tubuli samen en wordt het resterende filtraat als urine via de blaas uit het lichaam verwijderd (figuur 1). Uit de literatuur is bekend dat kleine eiwitten (met een gewicht kleiner dan 20 kDa) gemakkelijk door het filter van de nier gaan. In het eerste deel van de tubulus worden deze eiwitten vervolgens door de tubuluscellen opgenomen en in een bepaald deel van de cel (lysosoom) afgebroken tot hun bouwstenen, de aminozuren die voor hergebruik weer aan het lichaam worden teruggegeven.
Voor "drug targeting" naar de nier lijken kleine eiwitten geschikte geneesmiddeldragers. Wordt een geneesmiddel aan een klein eiwit gekoppeld dan zal dit geneesmiddel in de tubuluscellen terecht komen. Vervolgens zal tijdens de afbraak van het zogenaamde geneesmiddel-eiwit conjugaat, het actieve geneesmiddel in de tubuluscel vrijkomen (figuur 1, links).
Voor "drug targeting" naar de blaas zouden kleine eiwitten kunnen worden gebruikt die wel door het filter heengaan maar niet door de tubuluscellen worden opgenomen. Voor opname van een eiwit in de tubulus blijkt het belangrijk dat het eiwit positief geladen groepen heeft. Verwacht wordt daarom

dat "drug targeting" naar de blaas kan worden verkregen door het geneesmiddel te koppelen aan een klein eiwit waarvan de positieve groepen zijn afgeschermd (figure 1, rechts).



Figuur 1: *Transport van een in de arm gespoten geneesmiddel gekoppeld aan een klein eiwit. (grijs gearceerd)*
a: nier, b: filter (glomerulus), c: tubuluscel, d: lysosoom, e: blaas.

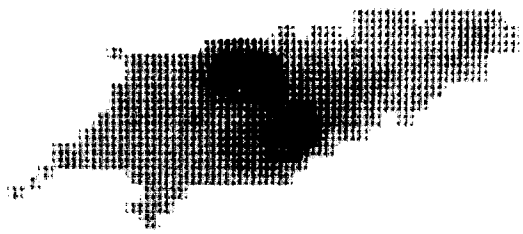
Worden kleine eiwitten voornamelijk in de nieren opgenomen en in de nieren afgebroken?

Hoofdstuk 2 en 3.

Van een drietal kleine eiwitten werd bepaald hoe ze in de gezonde rat worden verwerkt. Na intraveneuze toediening bleken alle drie eiwitten snel uit de bloedcirculatie te verdwijnen en voornamelijk in de nieren te worden opgenomen (figuur 2). Behalve in de nieren bleek er geen eiwit elders in het lichaam op te hopen. Onderling bleken er grote verschillen te zijn in de snelheid waarmee ze in de nier werden afgebroken.

De resultaten geven aan dat kleine eiwitten waarschijnlijk goed te gebruiken zijn als nier-specifieke dragers van geneesmiddelen. Het geneesmiddel-eiwit conjugaat zal snel in de nieren worden opgenomen zonder ongewenste opname elders in het lichaam. Afhankelijk van de binding tussen het geneesmiddel en het eiwit en van de afbraaksnelheid van het drager-eiwit, zal een geneesmiddel met een bepaalde snelheid in de nier vrijkomen hetgeen bepalend zal zijn voor de werkingsduur van het geneesmiddel in de nier.

Uitvoering experiment: Om de verwerking van een klein eiwit in het lichaam van de rat te kunnen volgen werd radioactief jodium aan het eiwit gekoppeld. De rat werd onder narcose gebracht en het radioactieve eiwit in een bloedvat ingespoten. De radioactiviteit in de rat werd gedurende een aantal uren continu met een gamma-camera gemeten: zie figuur 2. De nieropname en afbraaksnelheid van het eiwit konden vervolgens worden bepaald door in de loop van tijd de hoeveelheid radioactiviteit in het niergebied te vergelijken met de hoeveelheid in de totale rat.



Figuur 2: *Lichaamsverdeling van een radioactief klein eiwit in de rat:* representatieve opname van de gamma-camera 20 minuten na injectie van het eiwit in de bloedbaan.

Wordt de verwerking van kleine eiwitten in de nier beïnvloed door eiwitverlies via de nieren?

Hoofdstuk 4

Veel nierziekten gaan gepaard met eiwitverlies in de urine (proteinurie). Proteinurie wordt veroorzaakt door schade aan het filter (de glomerulus), waardoor niet alleen kleine maar ook grote eiwitten worden doorgelaten. Dit zou een probleem kunnen zijn voor "drug targeting" naar de nier met behulp van kleine eiwitten. Er zou namelijk competitie kunnen optreden tussen de gelekte eiwitten en het geneesmiddel-eiwit conjugaat in de opname en/of afbraak in de tubuluscellen van de nier. Om dit probleem in kaart te brengen werden ratten met een stof ingespoten waardoor ze proteinurie kregen. In deze ratten is vervolgens de opname en afbraak van kleine eiwitten in de nier

bepaald met behulp van de gamma-camera. De opname in de nier bleek niet beïnvloed door proteinurie. Echter, de snelheid waarmee de eiwitten in de nier werden afgebroken bleek lager bij ernstige proteinurie. Voor "drug targeting" betekent dit dat de afgifte van het geneesmiddel aan de nier intact zal blijven maar dat het geneesmiddel misschien minder snel zal worden geactiveerd tijdens proteinurie.

Targeting van naproxen naar de nier

Niet-steroide ontstekingsremmers zoals naproxen zijn therapeutisch effectief in de nier maar hebben een aantal schadelijke bijwerkingen elders in het lichaam. Om naproxen specifiek in de nier af te leveren werd het geneesmiddel aan het kleine eiwit lysozyme gekoppeld.

Hoe gedraagt naproxen-lysozyme conjugaat zich in de rat?
Hoofdstuk 5.
De koppeling aan naproxen bleek geen invloed te hebben op de verwerking van lysozyme in de rat. Het naproxen gekoppelde lysozyme werd, net als gewoon lysozyme, snel in de nieren opgenomen en vervolgens afgebroken. Koppeling aan lysozyme had wel een groot effect op de verwerking van naproxen. Ten opzichte van vrij naproxen werd na toediening van het naproxen-lysozyme conjugaat veel meer naproxen in de nier gevonden. Na de opname van het conjugaat in de nier nam de concentratie naproxen in de nier langzaam af in tijd. Dit is een aanwijzing dat het conjugaat wordt afgebroken en het naproxen vrijkomt in de nier. Tot slot was er na toediening van het naproxen-lysozyme conjugaat geen aantoonbare hoeveelheid vrij naproxen in het bloed aanwezig. Dit is een aanwijzing dat de koppeling aan lysozyme zal leiden tot de gewenste vermindering in naproxen activiteit elders in het lichaam.

Uitvoering experiment: de verwerking van het lysozyme-deel van het conjugaat werd bepaald met de gamma-camera zoals eerder beschreven. Omdat naproxen niet aan radioactief jodium kan worden gekoppeld is een meting met de gamma-camera niet mogelijk voor de bepaling van de verwerking van het naproxen-deel in de rat. Ratten werden onder narcose gebracht en ingespoten met het conjugaat. Op verschillende tijden na de injectie werden de ratten dood gemaakt en de nieren geprakt. In de geprakte nieren werd vervolgens de hoeveelheid naproxen bepaald. De opname en verdwijning van het naproxen deel van het conjugaat in de nier kon worden vastgesteld door in de loop van de tijd de hoeveelheid naproxen in de nier te vergelijken met de ingespoten hoeveelheid.

Veroorzaakt naproxen-lysozyme conjugaat effecten in de nier van de rat?
Hoofdstuk 6.
Na deze gunstige resultaten hebben we onderzocht of toediening van het naproxen-lysozyme conjugaat leidt tot effecten in de nier [hoofdstuk 6]. Naproxen is een ontstekingsremmer die de synthese van de zogenaamde prostaglandines remt. In de huidige studie hebben we bepaald of naproxen-lysozyme conjugaat toediening effect heeft op de prostaglandine uitscheiding via de urine van de gezonde rat. Alleen de toediening van een tamelijk hoge dosering vrij naproxen bleek effectief te zijn. Een vergelijkbaar effect werd verkregen na toediening van een veel lagere dosering naproxen gekoppeld aan lysozyme.

Uitvoering experiment: Voor deze studie kregen de ratten een permanent slangetje in een bloedvat. Dagelijks werd via dit slangetje het conjugaat aan de wakkere rat toegediend. De ratten werden op een rooster gezet met daaronder een urine opvangsysteem zodat urine continue kon worden verzameld voor de prostaglandine metingen.

Targeting van doxorubicine naar de blaas

Hoofdstuk 7.
Een specifieke afgifte van een cytostaticum via de urine aan de blaas kan interessant zijn voor de behandeling van blaastumoren. Toediening van cytostatica door middel van een injectie in het bloed is weinig zinvol omdat ze maar voor een klein deel via de urine worden uitgescheiden en omdat ze uitermate giftig zijn. Blaaskanker wordt daarom vaak behandeld door middel van blaasspoelingen met een cytostaticum hetgeen echter ook een aantal nadelen heeft. Wij beschrijven een nieuwe injecteerbare toedieningsvorm van het cytostaticum doxorubicine. Deze toedieningsvorm zou moeten leiden tot meer doxorubicine in de urine en minder of zelfs geen giftige activiteit van het doxorubicine elders in het lichaam. Doxorubicine werd aan het kleine eiwit lysozyme gekoppeld. Om de opname van het doxorubicine conjugaat in de tubuluscellen te voorkomen werden alle positief geladen groepen van het lysozyme gebruikt voor de koppeling met doxorubicine moleculen.

Wordt doxorubicine specifiek naar de blaas gebracht door het te koppelen aan het kleine eiwit lysozyme?
Toediening van doxorubicine gekoppeld aan het lysozyme leidde tot een 7-voudige toename van doxorubicine in de urine van de rat ten opzichte van vrij doxorubicine.

Uitvoering experiment: Bij deze studie kregen de ratten het doxorubicine-lysozyme conjugaat onder narcose ingespoten. Vervolgens werd gedurende een aantal dagen de urine verzameld voor de bepaling van doxorubicine uitscheiding.

Worden de schadelijke effecten van doxorubicine in de rat voorkomen door het te koppelen aan lysozyme?
In de rat zijn voornamelijk de nieren gevoelig voor de giftige werking van vrij doxorubicine. De ratten ontwikkelen proteinurie met microscopische afwijkingen aan de filters (glomeruli) in de nier. Bij de toediening van doxorubicine gekoppeld aan lysozyme bleken deze afwijkingen niet voor te komen. Zelfs een extreem hoge dosis van doxorubicine-lysozyme conjugaat had geen zichtbare beschadigende werking op de nieren van de rat.

Uitvoering experiment: Na een intraveneuze injectie van het doxorubicine-lysozyme conjugaat werd wekelijks een urine portie van 24 uur verzameld om de ontwikkeling van proteinurie in tijd te kunnen bepalen. Na 12 weken werden de ratten gedood en werd met de microscoop bekeken of de nieren waren beschadigd.

Toekomst

De hierboven beschreven studies laten een aantal mogelijkheden zien van "drug targeting" met behulp van kleine eiwitten. Geneesmiddelen kunnen specifiek naar de nier en blaas worden gebracht waarna het actieve geneesmiddel vrijkomt.
Voor vervolg onderzoek is het interessant om "drug targeting" met behulp van kleine eiwitten verder te ontwikkelen voor therapeutische doeleinden zoals de behandeling van chronisch nierfalen, acute nier insufficiëntie en tumoren aan blaas en nier. Verder kan het een belangrijk gereedschap zijn voor diagnostische doeleinden zoals de bepaling van nierdoorbloeding en lokalisatie van tumoren. Bovendien kan "drug targeting" worden gebruikt om meer inzicht te krijgen in de oorzaak van ziektes en de manier waarop geneesmiddelen werken.

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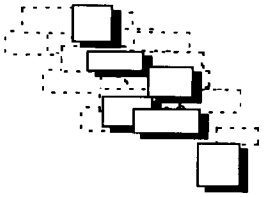
Dankwoord



Dankwoord

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28 januari zal het laatste steentje worden gelegd.
Iedereen is van harte welkom.

Gillian:
be there or be square!!